age than in the presence of ATP. In a separate set of measurements, neither fraction 2 nor insulin required the 50 μM Mg^{2+} normally present in the incubation to produce a stimulation of pyruvate dehydrogenase activity (data not shown).

The above results demonstrate that the factor isolated from insulin-treated muscle, which has been suggested by Larner *et al.* (*6*) to be the chemical mediator of insulin action, mimics insulin's activation of pyruvate dehydrogenase activity in a subcellular preparation from rat adipocytes. We identified the chromatographic fraction containing the insulin-generated factor from muscle as the one with the greatest activity reported by Larner *et al.* (*6*). This substance activated pyruvate dehydrogenase activity under incubation conditions similar to those under which insulin decreased phosphorylation of the alpha subunit of pyruvate dehydrogenase (*2*, *3*) and activated the enzyme (*4*). Both insulin and fraction 2 increased pyruvate dehydrogenase activity in the presence of Mg^{2+}, Ca^{2+}, and ATP and in the absence of ATP or Mg^{2+} and ATP. The effects of insulin on pyruvate dehydrogenase activity in the adipocyte subcellular system occurred only in the presence of both plasma membranes and mitochondria, whereas fraction 2 acted directly on mitochondria in the absence of plasma membranes. This suggests that insulin in this subcellular system generates a chemical mediator by its interaction with the plasma membrane and that fraction 2 may contain this substance.

The mechanism by which insulin and fraction 2, as well as concanavalin A and antibody to insulin receptor (*4*), stimulate pyruvate dehydrogenase activity appears to involve activation of a phosphatase activity and not inhibition of a kinase activity. This conclusion is based on the ability of these agents to act when no ATP is included in the incubation medium. Since the mitochondria themselves contain no measurable ATP (data not shown), these agents cannot be acting by inhibiting protein kinase activity. This is consistent with observations on the intact adipocyte which suggest that insulin stimulates pyruvate dehydrogenase by activating a calcium-sensitive phosphatase which dephosphorylates the alpha subunit of the enzyme leading to an increase in the active dephosphorylated form of the enzyme (*5*). This concept is supported by the observations of McDonald *et al.* (*7*) that insulin treatment of adipocytes did not alter total mitochondrial calcium but caused an increase in the labile form of the cation and a decrease in the stable form. This change in calcium pools could be responsible for activating the phosphatase. This model is also consistent with the demonstrated ability of the material in fraction 2 to stimulate phosphoprotein phosphatase activity (*6*).

Our studies support the suggestion that the factor isolated from insulin-treated muscle (*6*) is the chemical mediator for insulin action. Another possible candidate for this role, H_2O_2 (*8*), has been tested in the adipocyte subcellular system and found not to stimulate pyruvate dehydrogenase activity (data not shown). An interesting correspondence between the activity of the material in fraction 2 (*6*) and of insulin, concanavalin A, and antibody to insulin receptor in the adipocyte subcellular system (*4*) is their diminished effect at high concentrations. This behavior has also been reported for an insulin-dependent cytoplasmic material from liver which stimulates Ca^{2+} uptake by isolated mitochondria (*9*). Further studies are necessary to isolate and characterize the chemical mediator generated by insulin in the adipocyte subcellular system in order to determine whether it is identical to the insulin-generated material isolated from muscle (*6*) or that from liver (*9*), or both.

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References and Notes

1. J. R. Seals, J. M. McDonald, L. Jarett, *Biochem. Biophys. Res. Commun.* **83**, 1365 (1978).
2. ———, *J. Biol. Chem.* **254**, 6991 (1979).
3. ———, *ibid.*, p. 6997.
4. J. R. Seals and L. Jarett, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
5. D. L. Severson, R. M. Denton, H. T. Pask, P. J. Randle, *Biochem. J.* **140**, 225 (1974); D. L. Severson, R. M. Denton, B. J. Bridges, P. J. Randle, *ibid.* **154**, 209 (1976); R. M. Denton and W. A. Hughes, *Int. J. Biochem.* **9**, 545 (1978).
6. J. Larner, G. Galasko, K. Cheng, A. DePaoli-Roach, L. Huang, P. Daggy, J. Kellogg, *Science* **206**, 1408 (1979).
7. J. M. McDonald, D. E. Bruns, L. Jarett, *Biochem. Biophys. Res. Commun.* **71**, 114 (1976).
8. J. M. May and C. de Haën, *J. Biol. Chem.* **254**, 2214 (1979).
9. Ya. Kh. Turakulov, M. Kh. Gainutdinov, J. I. Lavina, M. S. Akhmatov, *Dokl. Akad. Nauk Uzb. SSR* **234**, 1471 (1977).
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Generation by Insulin of a Chemical Mediator That Controls Protein Phosphorylation and Dephosphorylation

Abstract. *Deproteinized skeletal muscle extracts free of major nucleotides from control and insulin-treated rats were fractionated and assayed for inhibition of protein phosphorylation by cyclic adenosine monophosphate (AMP)-dependent and -independent protein kinases. A differential effect of insulin on a particular fraction was observed on cyclic AMP-dependent protein kinase but not on cyclic AMP-independent protein kinases. This fraction that inhibited cyclic AMP-dependent protein kinase also stimulated glycogen synthase phosphoprotein phosphatase. It is proposed that this fraction may contain a mediator substance generated in the presence of insulin.*

A chemical intermediate in the mechanism of action of insulin was proposed to explain the dissociation of insulin stimulation of glucose transport and glycogen synthesis (*1*). In keeping with this hypothesis, we have established two separate biochemical mechanisms for the activation of glycogen synthase in the absence and in the presence of a transportable hexose (*2*). Prior to this work we had already established that insulin activated glycogen synthase with

Table 1. Inhibition of cyclic AMP-dependent protein kinase by column fraction 2 from Sephadex G-25 chromatography. Column fraction 2 from control and insulin-treated rat skeletal muscle extracts was lyophilized and redissolved in 1 ml of 0.05N formic acid. The reaction mixture for the protein kinase assay contained (total volume, 90 μl) 5 μl of inhibitor fraction, 8 mM $MgCl_2$, 120 mM morphoethanesulfonic acid (MES) buffer (pH 6.6), 0.12 mM [γ-^{32}P]ATP (800 to 1000 cpm/pmole), 0.40 mg of histone per milliliter and, when present, 2.5 μM cyclic AMP. After 10 minutes at 30°C, 15-μl portions were pipetted onto instant thin-layer chromatography strips (ITLC; Gelman) that were then spotted with 20 percent trichloroacetic acid, 1 mM ATP, and 4 mM P_i. Strips were then chromatographed in 5 percent trichloroacetic acid containing 0.2M KCl, and analyzed for radioactivity (*12*).

Cyclic AMP	Inhibition of protein kinase (%)		Net effect due to insulin (%)
	Control	Insulin	
None	45.6	60.4	33
Present	52.1	63.0	21

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no change in basal cyclic adenosine monophosphate (AMP) concentrations (*3*). We have also demonstrated that two high-molecular-weight materials each interacted in the insulin mechanism: (i) a pituitary diabetogenic peptide (25,000 daltons) which blocked the action of insulin, yet had weak agonist activity (*4*) and (ii) a human antibody receptor (150,000 daltons) which was indistinguishable from insulin in its agonist activity (*5*). From these experiments we argued that insulin acted initially at the cell membrane (*6*). Thus, it seemed reasonable to suppose that, as a result of the interaction, a mediator substance might be formed. To test this hypothesis, we used the cyclic AMP-dependent protein kinase as a bioassay since we (*7*) initially and others subsequently (*8*) had demonstrated that the protein kinase was present to a greater extent as a holoenzyme, which was decreased in enzyme activity, and in its capacity to bind and be activated by added cyclic AMP after insulin treatment (*9*).

In 1974 we first reported (*10*) that an acid- and heat-stable substance that inhibits the cyclic AMP-dependent protein kinase is generated early in insulin action. We subsequently showed that the inhibitor resulted in a decreased binding of cyclic AMP to the protein kinase (*6*, *9*). We now report additional purification steps, describe an initial characterization of the material, and show that it acts also to activate the phosphoprotein phosphatase that converts glycogen synthase to its active form. Jarett and Seals have demonstrated that this material, like added insulin, activates mitochondrial pyruvate dehydrogenase by dephosphorylation in a subcellular system in rat adipocytes (*11*). For these reasons we now term this material an insulin-mediator substance.

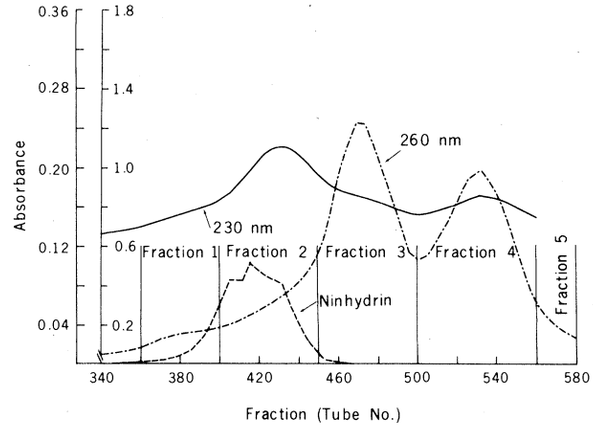

Fig. 1. Sephadex G-25 chromatogram of a skeletal muscle extract that had been deproteined and from which the major nucleotides had been removed by paper chromatography. Frozen muscle powder (25 g) was heat-treated, extracted, and chromatographed on paper. After elution, lyophilization, and reconstitution, 5 ml of the purified paper eluate was applied to the column; the column was washed twice with equal volumes of 0.05*N* formic acid and then developed with the same solution; 2.5-ml fractions were collected at a flow rate of 15 drops per minute in a fraction collector and then analyzed for absorbancy at 230 and 260 nm and for ninhydrin reactivity.

Rats were anesthetized with Nembutal for 15 minutes and then given insulin (4 U/kg) or saline intravenously. Hind leg muscle was removed 5 minutes later, and was rapidly frozen in liquid nitrogen. Blood samples were taken for glucose analysis. In the animals receiving insulin the mean blood glucose concentration decreased from 151.7 to 102.9 mg/dl, and muscle glycogen synthase was activated from 26.1 to 35.7 percent independent or I form. Frozen powdered muscle (25 g) was deproteinized by heating at 100°C for 3 minutes, adding two volumes of acetic acid (*p*H 3.8) containing 0.1 m*M* EDTA and 0.1 m*M* cysteine at 100°C, and heating for 4 minutes. The mixture was cooled on ice, centrifuged to remove particulate denatured protein, and filtered through glass wool; the clear yellowish supernatant was lyophilized. The tan powder was dissolved in 7.5 ml of 0.05*N* formic acid and streaked on washed Whatman 3MM filter paper sheets (46 by 57 cm); two papers were used for each 25 g of original muscle. Paper chromatography was performed with a mixture of 0.1*M* ammonium acetate and 95 percent ethanol (3:7) (*p*H 3.8) at room temperature in tanks flushed with nitrogen; the papers were then dried at room temperature. The adenosine triphosphate, diphosphate, and monophosphate (ATP, ADP, and AMP, re-

Table 2. Action of fraction 2d on cyclic AMP-dependent and -independent protein kinases. Sephadex G-25-fraction 2 (see Fig. 1) was chromatographed on thin-layer chromatography cellulose sheets in a mixture of ammonium acetate and ethanol (*p*H 3.8). Six fractions, a to f, were eluted with 0.05*N* formic acid, lyophilized, redissolved in 0.05*N* formic acid, and assayed with cyclic AMP-dependent and -independent protein kinases. Cyclic AMP-dependent protein kinase was assayed (as in Table 1), with homogeneous protein kinase from skeletal muscle (0.01 mg/ml). Independent protein kinases were assayed by the method described in Table 1 with the following modifications: The independent kinases were assayed at *p*H 6.8 while phosphorylase b kinase was assayed at *p*H 7.4. Glycogen synthase I (0.29 mg/ml) was substrate for all the independent kinases. Cyclic AMP-independent kinases were prepared as described (*14*). The enzyme PC 0.4 (phosphocellulose column fraction eluting with 0.4*M* KCl) was present at a final concentration of 0.18 mg/ml; PC 0.7 at 0.09 mg/ml. The reaction mixtures contained 0.5 m*M* EDTA and 0.2 m*M* EGTA; phosphorylase b kinase was added to a final concentration of 6.3 μg/ml and $CaCl_2$ to a final concentration of 1.2 m*M*.

Condition	Inhibition of protein kinase (%)		Net effect due to insulin (%)
	Control	Insulin	
Cyclic AMP-dependent protein kinase			
− Cyclic AMP	70.0	93.0	33
+ Cyclic AMP	38.0	74.0	95
Cyclic AMP-independent protein kinase			
PC 0.4	70.0	64.0	
PC 0.7	82.0	79.0	
Phosphorylase b kinase	69.0	68.0	

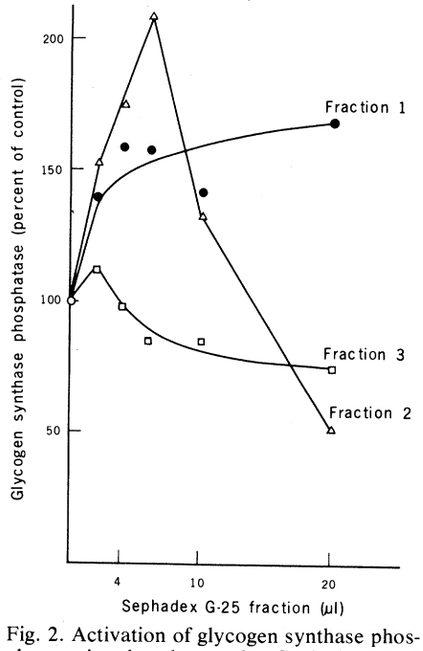

Fig. 2. Activation of glycogen synthase phosphoprotein phosphatase by Sephadex G-25 column fractions. Varying amounts of reconstituted fractions 1, 2, and 3 from insulin-treated rat skeletal muscle extract were lyophilized and redissolved in 0.2 ml of 140 m*M* MES (*p*H 7.0) containing glycogen synthase D (0.2 mg/ml) and a crude glycogen synthase phosphoprotein phosphatase (2.8 mg/ml). After incubation at 21°C for 20 minutes, 20-μl portions were removed for glycogen synthase assay in the absence and presence of glucose 6-phosphate. The reaction mixtures for the glycogen synthase assay contained (total volume, 90 μl) 4.4 m*M* uridine diphosphate glucose, 13.3 m*M* EDTA, 100 m*M* potassium fluoride, 6.7 mg of glycogen per milliliter, 7.2 m*M* glucose 6-phosphate, if present, and 33 m*M* tris buffer (*p*H 7.8). The mixtures were incubated for 10 minutes at 30°C and analyzed (*13*).

spectively) zones were identified and cut away. The remaining material was eluted with 0.05*N* formic acid and lyophilized. The dried material was taken up in 5 ml of 0.05*N* formic acid and chromatographed on Sephadex G-25 (column, 5 by 83 cm). Five fractions or peaks were identified (Fig. 1). There was evidence for peptide throughout the chromatogram from the absorbance at 230 nm and especially in fraction 2, where the major 230-nm peak corresponded with the ninhydrin-positive peak.

Molecular sieving of the biologically active ninhydrin fraction 2 indicates that its molecular size is between 1000 to 1500 daltons. Two and sometimes three 260-nm absorption peaks eluted later and were ninhydrin-negative. When these fractions were tested in the cyclic AMP-dependent protein kinase assay, inhibitory activity appeared in fractions 1 to 3; the major activity was in fraction 2, which also was the only fraction with increased inhibitory action of insulin, as compared to the control, whether the assay was performed in the presence or absence of cyclic AMP (Table 1).

Fractions 1 and 2 extracted from insulin-treated muscle also activated muscle phosphoprotein phosphatase in a dose-dependent manner, with fraction 2 being more potent (Fig. 2). At higher concentrations, fraction 2 had an inhibitory effect on the phosphatase. This effect may be due to the impure nature of the material. Fraction 3 was very weakly inhibitory. Further purification of fraction 2 by thin-layer chromatography (cellulose; ammonium acetate developer, and ethanol, *p*H 3.8) produced six fractions, which were assayed. Fraction 2d, which contained the inhibitor of the cyclic AMP-dependent protein kinase, demonstrated the difference between control and insulin in the absence, as well as in the presence, of cyclic AMP. To determine the specificity of the protein kinase inhibition, fraction 2d was tested on three different cyclic AMP-independent protein kinases, including phosphorylase b kinase. No difference between control and insulin was observed (Table 2), indicating that the material had specificity for the cyclic AMP-dependent protein kinase. Samples of all fractions separated by Sephadex G-25 column chromatography were lyophilized for activity on mitochondrial pyruvate dehydrogenase (*11*).

Because of its ability to mimic the action of insulin on cyclic AMP-dependent protein kinase, phosphoprotein phosphatase, and pyruvate dehydrogenase, we suggest that this peptide or peptidelike substance may constitute an insulin mediator. This material may be derived in some way from insulin or from the cell membrane, although we cannot exclude an intracellular origin.

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References and Notes

1. J. Larner, *Diabetes* **21** (Suppl. 2), 428 (1972).
2. J. C. Lawrence, Jr., and J. Larner, *J. Biol. Chem.* **253**, 2104 (1978); Y. Oron, G. Galasko, R. Cabelli, J. Larner, *Diabetes* **28**, 365 (1979).
3. N. D. Goldberg, C. Villar-Palasi, H. Sasko, J. Larner, *Biochim. Biophys. Acta* **148**, 665 (1967); J. W. Craig, T. W. Rall, J. Larner, *ibid.* **177**, 213 (1969).
4. T. B. Miller and J. Larner, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2774 (1972).
5. J. C. Lawrence, Jr., J. Larner, C. R. Kahn, J. Roth, *Mol. Cell. Biochem.* **22**, 153 (1978).
6. J. Larner, J. C. Lawrence, Jr., P. J. Roach, A. A. DePaoli-Roach, R. J. Walkenbach, J. Guinovart, R. J. Hazen, *Cold Spring Harbor Symp. Quant. Biol.*, in press.
7. L. C. Shen, C. Villar-Palasi, J. Larner, *Physiol. Chem. Phys.* **2**, 536 (1970).
8. O. Walaas, E. Walaas, O. Gronnerod, *Eur. J. Biochem.* **40**, 465 (1973).
9. R. J. Walkenbach, R. Hazen, J. Larner, *Mol. Cell. Biochem.* **19**, 31 (1978).
10. J. Larner, L. C. Huang, G. Brooker, F. Murad, T. B. Miller, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 261 (1974).
11. L. Jarett and J. R. Seals, *Science* **206**, 1407 (1979).
12. K.-P. Huang and J. C. Robinson, *Anal. Biochem.* **72**, 593 (1976).
13. J. A. Thomas, K. K. Schlender, J. Larner, *ibid.* **25**, 486 (1968).
14. A. A. DePaoli-Roach, P. J. Roach, J. Larner, *J. Biol. Chem.*, in press.
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Derived X Chromosome in the Turtle Genus *Staurotypus*

Abstract. *C-banding, G-banding, and silver (Ag-AS) staining techniques reveal a distinctive sex chromosome system in the turtle* Staurotypus salvinii. *Unlike previously described systems in most other vertebrate groups in which the Y or W is derived and the homogametic sex represents the primitive condition, the reverse is true for* S. salvinii. *The X chromosome is derived; thus the homogametic sex (female) is more derived than the heterogametic sex. The male is intermediate between the female and the ancestral condition observed in other turtle species.* Staurotypus *does not conform to the general model of sex chromosome evolution for diploid dioecious organisms.*

The only known case of heteromorphic sex chromosomes in turtles was reported by Bull *et al.* (*1*) for the genus *Staurotypus* (Kinosternidae; Staurotypinae); an XX/XY sex chromosome system in which the male is the heterogametic sex. Bull *et al.* (*1*) examined three male and two female *S. salvinii* and two male and one female *S. triporcatus*. In addition, we have examined one male and three female *S. salvinii*. The X is subtelocentric, with a secondary constriction on the long arm near the centromere, and the Y is acrocentric (Fig. 1). Evidence from meiotic pairing indicates these elements are homologous along most of their length except for a short, unpaired terminal segment, probably corresponding to the secondary constriction and short arm in mitotic preparations [figure 2, c and d, in Bull *et al.* (*1*)]. We now report our studies of this sex chromosome system with C-banding, G-banding, and silver (Ag-AS) staining techniques.

Metaphase chromosomes were harvested from fibroblast cell cultures initiated from heart biopsies as described for turtles (*2, 3*). G-bands were induced by the trypsin treatment (*4*), constitutive heterochromatin was stained by Sumner's procedure (*5*), and the ammoniacal silver (Ag-AS) technique (*6*) was used to locate the nucleolar organizer regions (NOR's). The last three pairs of chromosomes in the group B complement do not band as distinctly as the first two pairs (Fig. 1) (*2, 3*); but the long arm of the sex chromosome pair in *S. salvinii* appears to be homologous to the third group B pair in *Chinemys reevesi*. The G-band negative short arms are clearly visible on the X chromosomes (Fig. 1, B and C), while the secondary constriction on the long arm is best seen in a standard karyotype [figure 1 in (*1*)] or C-band preparation (Fig. 1D). The Y chromosome is acrocentric and homologous to an acrocentric pair in *Chinemys reevesi* (Fig. 1A) and several other turtle genera (*2, 3, 7*). Our C-band preparations of several *Staurotypus* cell cultures all showed that heterochromatin is restricted to the centromeric regions except for the X chromosome, in which the short arm, the centromeric region, and the secondary constriction region of the long arm are all heterochromatic (Fig. 1D). The Ag-AS staining shows that these secondary constrictions on the X chromosomes in *S. salvinii* contain the NOR's, the locations of genes coding for 18*S* and 28*S* ribosomal RNA (Fig. 2).

An interesting feature of the sex

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