IMMUNOBIOLOGICAL STUDIES IN TWO CASES OF INSULIN-RESISTANT DIABETES

Successful Corticoid Therapy in One Case With Progressive Acidosis, Without Detectable Circulating Antibodies. Antidiuretic Effect of Chlorpropamide In Another Case With Associated Diabetes Insipidus

LÍCIO MARQUES DE ASSIS, WILIAN NICOLAU, JOÃO BOSCO SALOMON, ANTONIO BARROS DE ULHÔA CINTRA, RÔMULO RIBEIRO PIERONI

PUBLICAÇÃO IEA N.º 200
Dezembro — 1969

INSTITUTO DE ENERGIA ATÔMICA
Caixa Postal 11049 (Pinheiros)
CIDADE UNIVERSITÁRIA "ARMANDO DE SALLES OLIVEIRA"
SÃO PAULO — BRASIL

IMMUNOBIOLOGICAL STUDIES IN TWO CASES OF INSULIN-RESISTANT DIABETES. Successful Corticoid Therapy In One Case With Progressive Acidosis, Without Detectable Circulating Antibodies. Antidiuretic Effect of Chlorpropamide In Another Case With Associated Diabetes Insipidus*

Lício Marques de Assis,
Wilian Nicolau,
João Bosco Salomon,
Antonio Barros de Ulhôa Cintra,
Rômulo Ribeiro Pieroni

Divisão de Radiobiologia
Instituto de Energia Atômica
São Paulo - Brasil

Publicação IEA Nº 200
Dezembro - 1969

* Separata da REVISTA DA ASSOCIAÇÃO MÉDICA BRASILEIRA - Vol. 16 - nº 1, janeiro - 1970.

Comissão Nacional de Energia Nuclear

Presidente: Prof.Dr. Hervásio Guimarães de Carvalho

Universidade de São Paulo

Reitor: Prof.Dr. Miguel Reale

Instituto de Energia Atômica

Diretor: Prof.Dr. Rômulo Ribeiro Pieroni

Conselho Técnico-Científico do IEA

Prof.Dr. José Moura Gonçalves	pela USP
Prof.Dr. José Augusto Martins	
Prof.Dr. Rui Ribeiro Franco	
Prof.Dr. Theodoreto H.I. de Arruda Souto	pela CNEN

Divisões Didático-Científicas

Divisão de Física Nuclear -
Chefe: Prof.Dr. José Goldenberg

Divisão de Radioquímica -
Chefe: Prof.Dr. Fausto Walter de Lima

Divisão de Radiobiologia -
Chefe: Prof.Dr. Rômulo Ribeiro Pieroni

Divisão de Metalurgia Nuclear -
Chefe: Prof.Dr. Tharcísio D.S. Santos

Divisão de Engenharia Química -
Chefe: Lic. Alcídio Abrão

Divisão de Engenharia Nuclear -
Chefe: Engº Pedro Bento de Camargo

Divisão de Operação e Manutenção de Reatores -
Chefe: Engº Azor Camargo Penteado Filho

Divisão de Física de Reatores -
Chefe: Prof.Dr. Paulo Saraiva de Toledo

Divisão de Ensino e Formação -
Chefe: Prof.Dr. Rui Ribeiro Franco

IMMUNOBIOLOGICAL STUDIES IN TWO CASES OF INSULIN-RESISTANT DIABETES[1]

Successful Corticoid Therapy In One Case With Progressive Acidosis, Without Detectable Circulating Antibodies. Antidiuretic Effect of Chlorpropamide In Another Case With Associated Diabetes Insipidus.

Lício Marques de Assis*, M.D.
Wilian Nicolau*, M.D.
João Bosco Salomon*, M.D.
Antonio Barros de Ulhôa Cintra**, M.D.
Rômulo Ribeiro Pieroni***, M.D.

SUMMARY

Clinical and immunological studies of two patients with marked insulin resistance are reported. In the first case, the striking and life-saving effect of corticoid suggested that resistance to insulin could be related to an immunobiological mechanism. However, no neutralizing antibody could be detected in the circulating blood. The puzzling question of the possible existance of a mechanism of neutralization at cellular level is discussed. In the second case neutralizing or blocking antibody (against exogenous insulin) could be detected in the blood during the resistant phase by a radioimmunotechnique. Disappearance of the clinical resistance coinciding with the administration of ditiazanine iodide (a poor soluble diazine) and hypoglicemic drugs (chlorpropamide), was followed by a striking change in the electrophoretic pattern of commercial labelled insulin. This new pattern gave supportive evidence that both drugs were able to destroy or inactivate the antibody or simply to promote the cleavage of the insulin-antibody complex and decrease resistance to the action of exogenous insulin. The marked and persistent reduction of the urinary daily volume, which was out of proportion with the diabetic condition, has been explained by the antidiuretic effect of chlorpropamide on true diabetes insipidus.

Different types of insulin resistance have been reported in the literature. As far as 1926, Mauriac and Aubertin[(1)] suggested that, beside infections, coma or other stresses and factors associated with hypophysis, adrenal, thyroid or liver, there could

---

1. Estudos imunobiológicos em dois casos de diabetes insulino-resistentes. Terapêutica corticóide bem sucedida em um caso com acidose progressiva, sem circulação detectável de anticorpos. Efeito antidiurético da clorpropamida em outro caso com diabete insípido associado. / Études immunobiologiques en deux cas de diabète insulino-résistants. Succès de la thérapie corticoide dans un cas avec acidose progressive, sans circulation détectable d'anticorps. Effet antidiurétique de la chlorpropamide dans un autre cas avec diabetes insipidus associé. Apres. para pub. em 16/10/69; aprov. em 24/11/69.

* Endocrinologie and Metabolic Unit, Hosp. das Clín., Fac. of Med., Univ. S. Paulo, Brasil; Research Physician, Inst. of Atomic Energy, S. Paulo.
** Prof. of Med., Fac. of Med., Univ. S. Paulo.
*** Chief of the Radiobiol. Division of the Inst. of Atomic Energy, S. Paulo

be mechanims of neutralization of insulin which, de per se, would be responsible for the onset of diabetes. After introduction of insulin , unusual cases of high resistance to treatment, fortunately rare, were observed. In some patients[2,8] the requirement may exceed several hundreds or even thousands of the current daily dosage. The existance of an immunobiologic mechanism was discovered and the presence of anti-insulin antibodies detected by a method first used by Banting et al.[9] in a case of diabetes resistant to the hormone. This fact led to the introduction of new and ingenious methods of research aiming to direct detection of substances, (probably anti-insulin antibodies) in serum of patients previously treated with insulin [10,12]. Burrow et al. in 1957[13] were able to demonstrate in cases of insulin-resistant diabetes (but not in normal and non-resistant) that insulin forms in serum a complex which migrates in the leading edge of gamma-globulin fraction on electrophoretic paper.

The opportunity we had to follow two cases of high resistance to insulin and peculiar response to treatment respectively with corticoids and ditiazanine iodide, led us to some interesting immunologic research.

CASES REPORT

Case I - R.P., male, white, catholic priest, 49 years old, teacher, from Campos, Rio de Janeiro. Admitted to the Hospital on March 15, 1960. His symptoms began in 1950. He lost 4 Kg weight in the first fours years. Since 1954 he suffered from dizziness, weakness and fatigue at work, feeling better, however, on more active life. Treatment with regular insulin, 20 units before each meal, started in 1954. During this year he respond end well to insulin treatment and carbohydrate restriction, remaining free of symptoms, but for mild sporadic hypoglicemic crises. In 1955 he abandoned treatment until Jan. 1959, in spite

of eventual moderate polyuria and polydipsia. At this time, dizziness, weakness and fatigue at work reappeared. These symptoms were markedly aggravated by emotional upsets. Insulin treatment was resumed in doses of 30-35 units of NPH daily. In Oct., after an episode of diarrhoea lasting three days, he began to lose weight and experienced intensification of the symptoms. In February, 1960, in spite of insulin treatment, the patient had an episode of ketoacidosis, "high" doses of insulin and saline infusion being necessary to correct the metabolic disturbance. One week later a new and more intense crisis of ketoacidosis and coma required "much higher" doses of insulin. Then the patient learned how to recognize the onset of acidosis because of pyrosis and marked dryness of mouth and throat. On these occasions he increased the total amount of insulin up to 180 units per day, preventing the aggravation of the disease. To try a better control of diabetes the patient came to S. Paulo. His past medical history is noncontributory. The parents and twelve siblings were in good health. Two paternal uncles had died and the cause of death was attributed to diabetes. Since the beginning of symptoms he complained of poor vision. On physical examination he appeared in good general conditions and well nourished. He weighed 70 kg and was 1.75m tall. Blood pressure 170 x 98mm Hg. Neurologic examination revealed absence of the knee and ankle jerks. Fundi showed moderate retinopathy and mild vascular lesion. Urinalysis from a sample collected at admission revealed no ketonic bodies. Sugar 4+*. Blood glucose 280mg per 100ml. (Folin-Wu). He was given 160 units of regular insulin in four separate doses from March 15 to 16. The total urine volume was 6.620 ml with a total sugar loss of 367g. Fasting blood sugar on March 16 was 292mg per 100ml. On march 16 the insulin was increased to 200 units in four divided doses. At noon, just one hour after the second injection, he felt worse, complaining of malaise and presenting deep breathing and psychomotor restlessness. He was

*, Clinitest

then given 100 units of regular insulin aubcutaneously, later intravenously (i.v.) each hour, and continuous infunsion of fluids (fig. 1). Because of persistence of ketonuria and aggravation of symptoms the doses of insulin were increased to 200 units i.v. in progressively more frequent injections; the patient received a total of 2,250 units in seven hours. From 2.30 P.M. to 6.25 P.M. he received 2,600ml of fluids (as isotonic glucose lactate and saline); infunsion was temporarily interrupted because of occurrence of pulmonary edema and resumed slowly a few minutes later . Despite all these measures, acidosis persisted and the general condition of the patient became progressively worse. Finally he fell into a frankly psychotic state (disorientation, delirium, hallucination and delusion). 100mg of cortisol hemisuccinate added to a final fluid infusion, during eighteen minutes at a rate of 5,5ml per minute, led to marked improvement of his condition fifty minutes later: consciousness was rapidly regained, he became calm, ketonuria dropped to 2 + or 1 +. Insulin was reduced to 50 units i.v. each half hour. On March 17 the patient received 360 units i.v. and 75 units s.c. Ketonuria disappeared at 11.30 A.M. and glycosuria was absent at 6 P.M. The patient remained mentally well. About 38 hours after the administration of cortisol there was a relapse, with growing intensity, of glycosuria and ketonuria, leading to increase of insulin requeriment. On March 18 the patient was given 50 units of NPH and 50 units of Regular insulin. On March 19 the administration of 100 units NPH and 300 units of Regular failed to prevent ketoacidosis (fig. 2). A second i.v. injection of 100mg of cortisol was followed again by improvement in symptoms and reduction in glycosuria and disappearance of ketonuria. A tendency to a new relapse of ketoacidosis occurred 30 hours later. The patient was then given 20mg daily per os of prednisone in four divided doses (fig. 3). Rapid weight loss was then observed coinciding with elimination of fluids accumulated during hyperhydration (fig. 3). Two weeks after the beginning of corticotherapy (April 4)

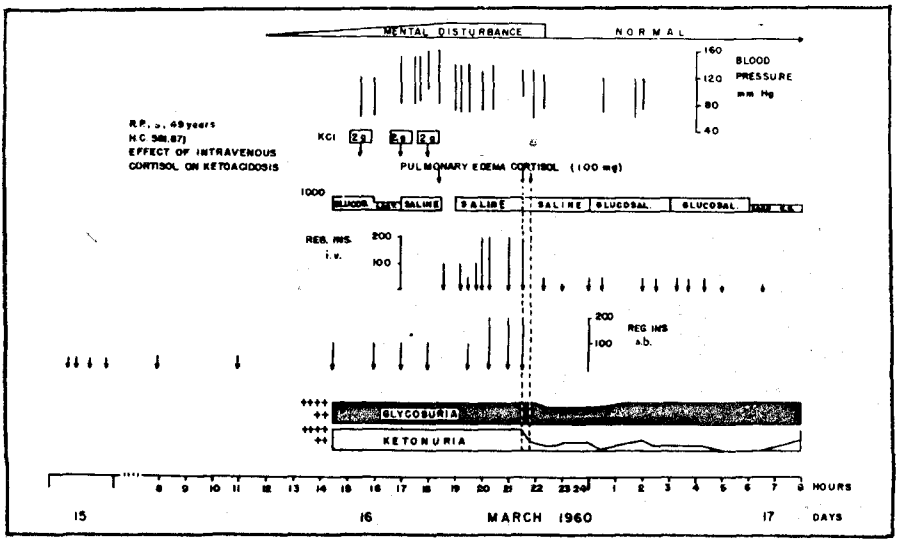


Fig. 1 - Influence of cortisol, as hemisuccinate, added to a final fluid infusion, during eighteen minutes, on the clinical and metabolic course of ketoacidosis. Note the abrupt decline in the ketonuria and glycosuria and disappearance of mental disturbance.

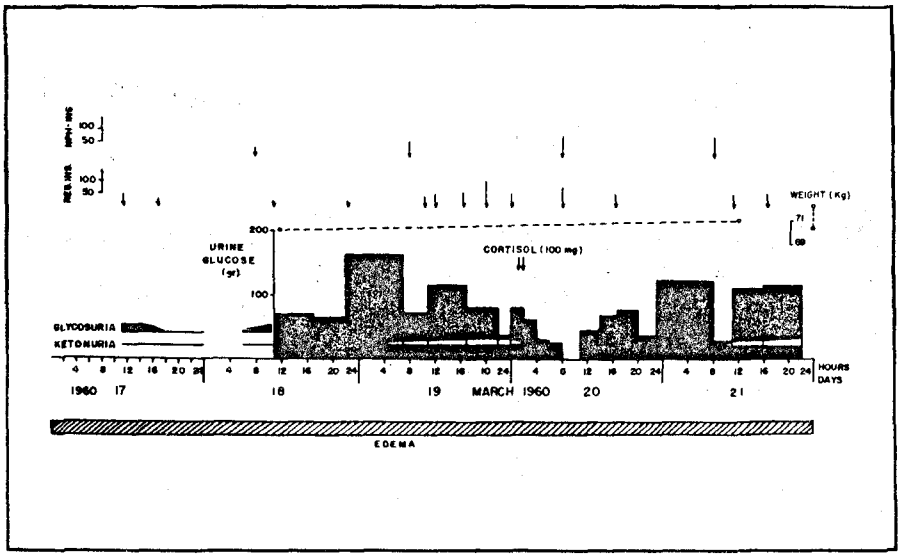


Fig. 2 - On March 19, the administration of 100 units NPH and 300 units Regular insulin failed to prevent ketoacidosis. A second intravenous injection of 100 mg of cortisol was followed again by improvement in symptoms, reduction in glycosuria and disappearance of ketonuria.

it was decided to mantain a constant dose of insulin (100 units NPH before breakfast and 150 units of regular insulin respectively at 11.00 A.M. and 6.00 P.M. before meals). Under this treatment

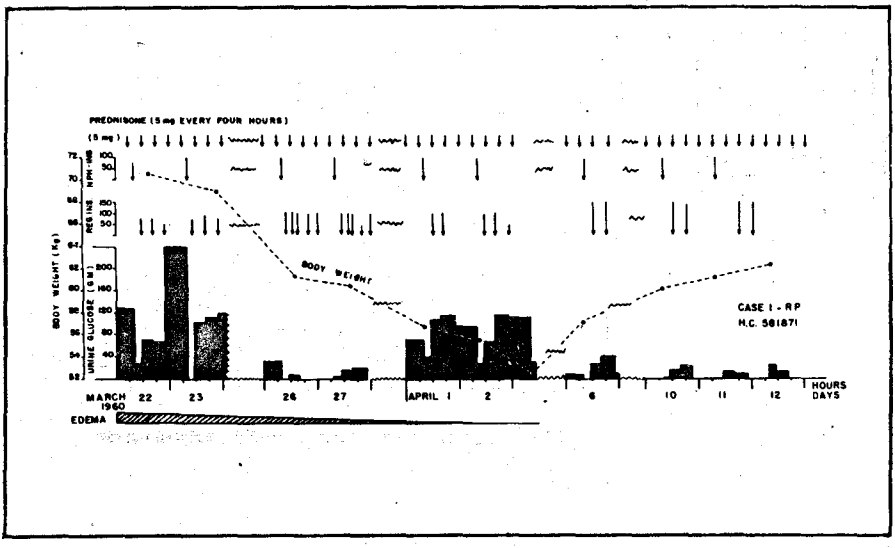
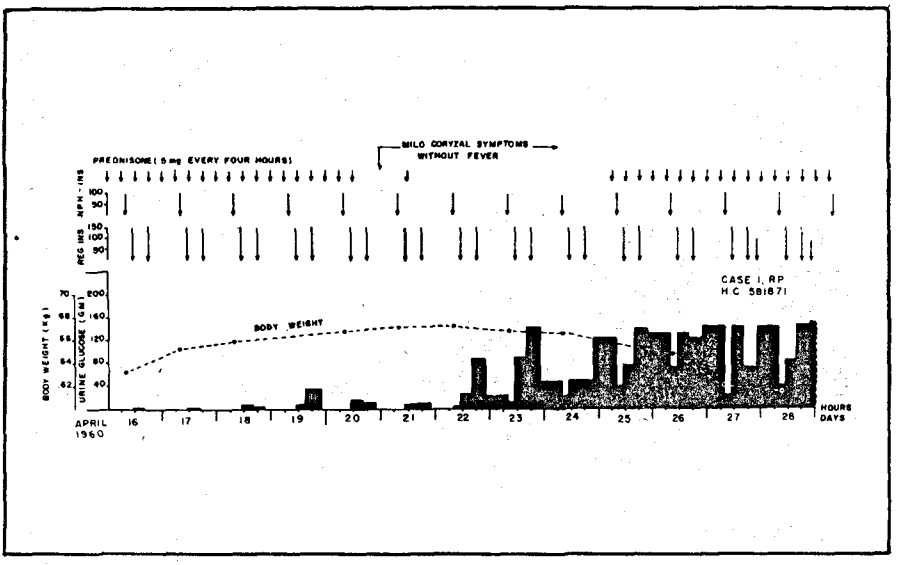


Fig. 3 - During prednisone administration a rapid weight loss was observed, coinciding with elimination of fluids accumulated during hyperhydration. The need for insulin fell from 650 to 300 units per day; glycosuria came down to relatively low level and the weight increased progressively without edema.

and a diet consisting of 398 gm of carbohydrate, 100 gm of protein, 108 gm of fat and a total daily caloric intake of 2.970 calories, glycosuria persisted on a relatively low level, being present after meals and absent at night. The actual beneficial effects of steroid on the evolution of diabetes was clearly proved on two different occasions, when the prednisone was intentionally discontinued: from April 21 to 24 (fig. 4) and May 12 to 16 (fig. 5); the total glycosuria rose, the weight fell and intensification of diabetic symptoms was observed. It seems advisable to stress that diet, doses and intervals of administration of insulin were not changed. On discharge the patient was receiving 100 units NPH and 150 units regular insulin before meals daily, and 10mg of prednisone every 12 hours. He was oriented to maintain basically the same therapy and diet at home. Re-examination of the patient in the Out-Patient Clinic has been done periodically. During the first year after discharge the patient referred marked redution in insulin requirement and, periodically, reduced and at times totally suppressed the prednisone



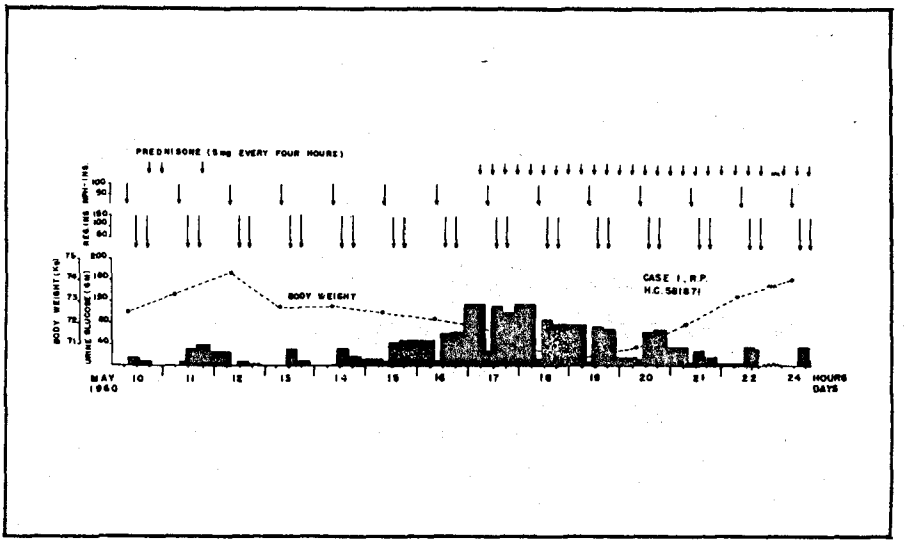


Fig. 4 and 5 - From April 21 to 24 and May 12 to 16, the interruption of prednisone therapy was followed by a rising in glycosuria, a plateau or tendency for decreasing weight and intensification of diabetic symptoms.

therapy. Finally, the need for prednisone disappeared and since 1963 he has made no use of this steroid. On recent examination (11/23/66) he was fairly well controlled of diabetes, but urea

was somewhat elevated (84mg/100ml) and blood pressure higher (180 x 100mm Hg). The weight was inaltered. The approximate need for insulin was 40 to 80 units NPH per day.

Immunoassay methods and results

Blood was collected six days after prednisone withdrawal (5/17/60), about 12 hours after the last regular insulin injection, to investigate anti-insulin antibodies eventually present in the serum of the patient, in an attempt of clarifying the mechanism of resistance. According to the Banting method[9] starved mice were injected with serum mixed with an amount of insulin wich, when injected with normal serum (or saline) caused convulsions in 90-100 per cent of the mice. The serum of the patient showed no neutralizing activity. The hemagglutination and gel-diffusion tests were also negative. Finally, the radioimmunotechnique[12,13] was performed for detection of anti-insulin antibody. Commercial insulin* was labelled with ^{131}I according to Banks technique[14] modified by Burrows el al.[13]. Carrier-free ^{131}I was supplied by the Institute of Atomic Energy, S. Paulo, Brazil. Labelled insulin had low specific activity and the total insulin per ml was always more than 10mg - 0,05 ml of labelled insulin were incubated with 0.200ml of normal and diabetic serum. After a few minutes of incubation at room temperature 0.02 ml of each mixture and the insulin solution were applied on electrophoretic paper. Total amount of insulin applied on the origin of the electrophoretic strip was always over 50 micrograms. Horizontal open strip method employing conventional type of equipment has been used for electrophoresis at room temperature, in buffer veronal, pH 8.6, ionic strenght 0.05. The types of filter paper for electrophoretic development are specified in figures. Electrophoretograms were dried in an oven at 120 C for 10 minutes and then stained with amidoschwartz 10 B for 20 minutes. When 0.02 to 0.04 ml of the solution of labelled insulin was applied, the electrophoretogram eventually show

* Crystalline Zinc Insulin, ARG-44-B, Lilly.

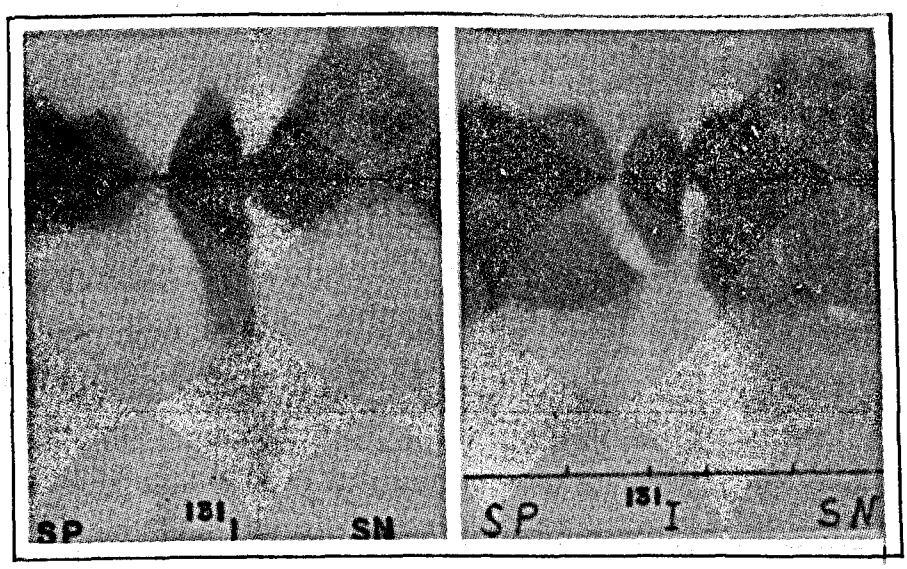


Fig. 6 - a) The radioautogram obtained with 16 hours electrophoresis strip after 19 days hours electrophoresis strip after 19 days contact with X-ray film. Note that insulin -^{131}I alone or mixed with normal serum (SN) migrates in the α1-albumin zone on Whatmann nº 1 paper. In mixture with the patient serum the labelled insulin shows the same mobility indicating probable absence of neutralizing antibodies in circulating blood. b) Electrophoretogram stained with amidoschwartz to show the albumin and γ-globulin zone after 16 hours electrophoresis.

ed a spot. This was due to human albumin used to prevent adsortion of labelled insulin in the glassware during the process of preparation. Fig. 6a shows the radioautogram obtained with 16 hours electrophoretic strip after 19 days contact with X-ray film. Insulin ^{131}I alone and in mixture with normal serum were used as controls. Fig. 6b shows the electrophoretogram stained with amidoschwartz. Insulin-^{131}I with low specific activity, mixed with normal control serum migrates in the α_1-albumin zone on electrophoretic paper Whatmann nº 1. According to Kallee[15], amounts of insulin lower than 0,2 µg, applied on Whatmann electrophoretic paper remain at the site of application whether or not in mixture with the serum. By adding an excess of stable insulin due to the saturation of the sites of application, the labelled insulin moves in the albumin or pre-albumin zone. In mixture with the patients serum, the labelled insulin showed the same mobility indicating probable absence of neutralizing antibodies in circulating blood[13]. Figure 7 shows a

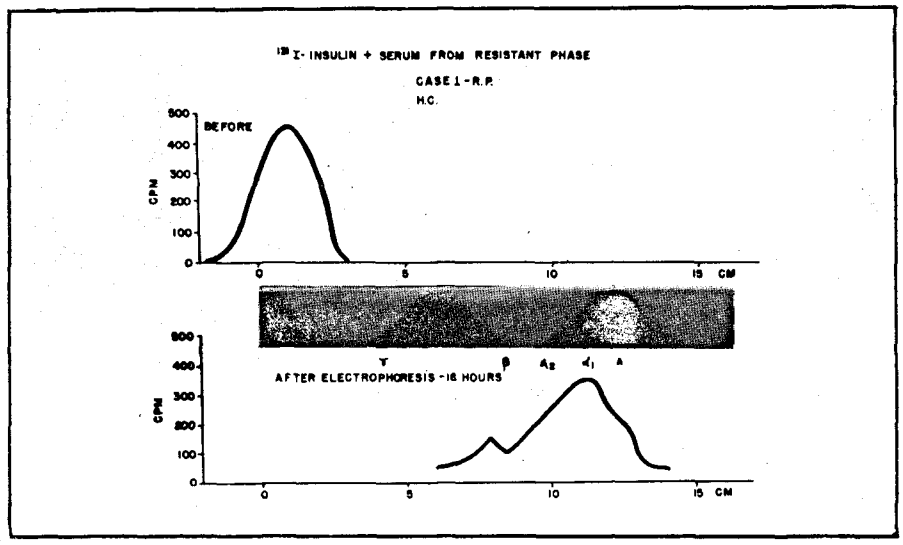


Fig. 7 - The activity was measured by an autoscanner before and after 16 hours electrophoresis of the same serum, whose radioautogram was shown in fig. 6. Experiment was performed three years later. The serum was stored frozen. As shown in the figure the labelled insulin migrates in the α_1-albumin zone. Insulin-^{131}I alone (fig. 10) and mixed with normal serum (fig. 11) were used as controls of this experiment.

second experiment made on the same stored frozen serum, three years later. Insulin-^{131}I was prepared by the same method, the activity being measured by an autoscanner before and after 16 hours of electrophoresis on Whatmann nº 1 filter paper. As shown in the figure the labelled insulin migrates in the α_1-albumin zone. Insulin-^{131}I alone (fig. 10) and mixed with normal serum (fig.12) were used as controls.

Case II - I.F.D.F., married, white, 48 yeras old woman, admitted on June 13, 1960. She was known to have diabetes since early 1952. After a few days of low dosage of insulin she abandoned tratment, remaining free of symptoms until 1959. Relapse of symptoms (polyuria, polydipsia, vulvar pruritus) occurred on May 1959. On this occasion symptoms subsided after one month of treatment with 20 units NPH insulin; she continued treatment with chlorpropamide (250mg/daily) without dietary re-

strictions. The diabetes was well controlled until May,1960. Tests for glycosuria, made periodically, were negative. Just one month prior to admission she complained of abrupt marked rise in diuresis, insatiable thirst, losing 10 to 20 liters of urine per 24 hours. In view of negative tests for glycosuria the diagnosis of diabetes insipidus was suspected. She was then admitted in the ward. Her past medical and familiar history is noncontributory. On physical examination she appeared well nourished. She weighted 71 Kg and was 1,64 m tall. Blood pressure 180 x 105mm Hg. Electrocardiogram revealed slight changes in ventricular repolarization. Cataract was present at the right, and slight narrowing of arterioles was seen in the left eye. Water restriction test starting in the morning led the patient to lose 4 per cent of her body weight in six hours and maximal specific gravity was 1,004. On Sept. 23, the patient was discharged under 5 units of vasopressin daily, with an average diuresis of 2 liters of ..... aglycosuric urine. On January 12, 1961, because of a positive test for glycosuria, the patient was placed on a low carbohydrate diet and 250 mg of chlorpropamide daily. Vasopressin tanate was maintained. On January 28 she was losing 170g of glucose per day and NPH insulin was substituted for chlorpropamide in doses of 30 units per day. On February 2, the insulin was increased to 40 units daily. From March 10, 1961 until Nov. 1962 she had been again fairly well controlled under chlorpropamide alone; during this interval hydrochlortiazide was substituted for vasopressin. The mean diuresis during this period was 5 liters and glycosuria fluctuated between 20 to 40 gr per day. A relapse of symptoms occurred during Nov. 1962. On Nov. 7 urinalysis revealed 284 gr of glucose in a 8 liters volume per 24 hours. Chlorpropamide and hydrochlortiazide were suppressed and she was given progressively higher doses of NPH insulin, from 20 up to 60 units per day. On February 11, 1963 the patient was admitted again. Under low solutes and high carbohy-

drate diet (Kempner's rice diet consisting of 453 gm of carbohy - drate, 27,3 gm of protein, 2,4 gm of fat and 1.39 mEq of sodium) from Feb. 25 to April 13 (figura 8) water volume fluctuated between 7,100 and 14,900 ml, averaging approximately 10,000ml per day. Weight increased slightly. Glycosuria remained, in general , below 100g per day. She was receiving previously 70 units NPH insulin at breakfast and 40 units Regular insulin in two divided doses, before meals. These doses were increased progressively to 80 and 90 respectively. From April 14 to June 17, under standard but hyposodic diet (336gm of carbohydrate, 89.7gm of protein, 85.8gm of fat and 19mEq of sodium) yielding large solute load, urinary volume rose, fluctuating between 12,750 and 22,975ml, except on the 15th, 16th and 17th May, when vasopressin was administered. During this period, excluding those three days, the mean urine volume was approximately 15,000 ml per day. Glycosuria rose markedly, attaining values above 400gm per day. From May 8 on , the patient was receiving a total of 190 units of insulin,clearly inadequate for compensating her diabetes. Suspition of increasing resistance to insulin due to antibody mechanism led to the collection of blood for immunoassay. Blood was then collected 24 hours after discontinuance of insulin injection (R_1, 6/7/63).From 22th June to 14th July the patient was put on rice diet again. Due to the presence of Strongyloids stercoralis in the faeces the patient was given ditiazanine iodide, 600mg per day,during twelve days. A surprisingly abrupt decrease in urinary volume and ..... glycosuria was then observed (fig. 9); the former fell from 12,000 to below 4,000ml and glycosuria disappeared. Insulin was then suppressed. Blood was collected for antibody assay when glycosuria and water diuresis were minimal (S_1, 7/12/63). From 15th July on, the patient received standard diet. Urinary volume and glycosuria rose again above 18,000 ml and 300gm respectively. A second resistant blood serum sample wasdrawn on 8/9/63 (R_2). A new period of ditiazanine iodide therapy was attempted, the patient being

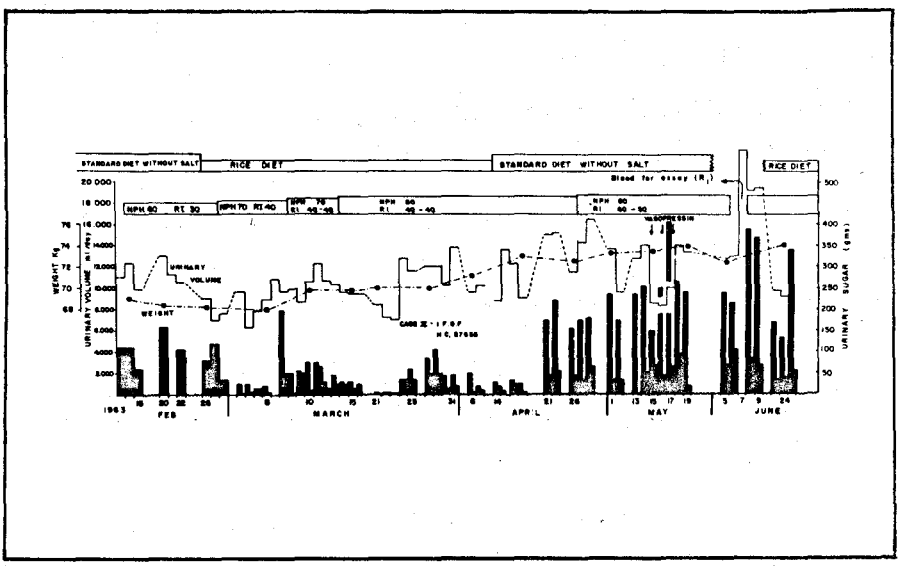


Fig. 8 - Under low solutes and high carbohydrate regimen water volume fluctuated between 7,100 and 14,900 ml per day. Glycosuria, in general, remained below 100gm per day. Glycosuria, in general, remained below 100gm per day under large solute load, urinary volume rose, fluctuating between 12,750 and 22,975 except on the 15th, 16th and 17th May when the patient received vasopressin injections. Glycosuria increased markedly attaining values above 400 gm per day. The need for insulin increased progressively. In spite of receiving a total dose of 190 units of insulin the patient lost more than 200 gm of glucose per day. On 6/7/63, 24 hours after the last insulin injection, blood was drawn for immunoassay (serum R_1).

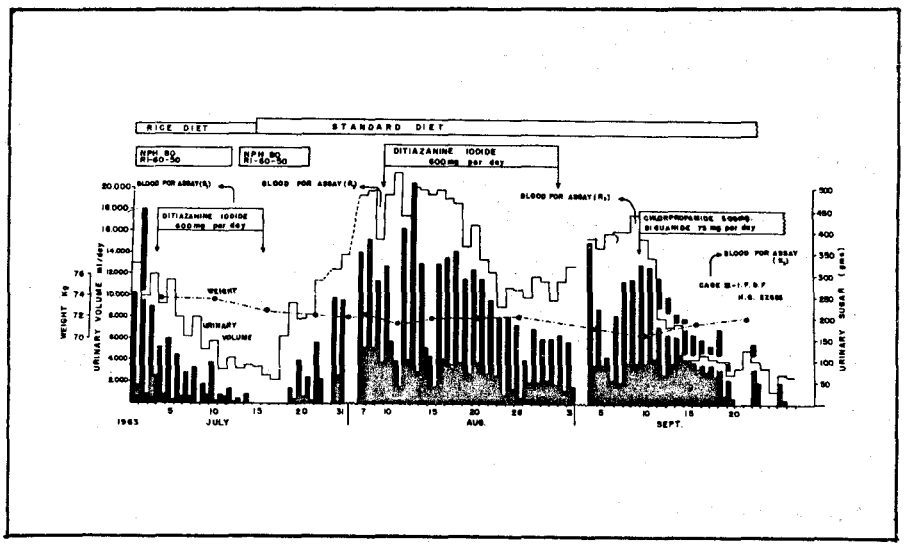


Fig. 9 - From July 4 to 15 the patient received ditiazanine iodide due to the presence of Strongyloids stercoralis in the faeces. Note the abrupt decrease in urinary volume and glycosuria; the former falls from 12,000 to below 4,000 ml and glycosuria desappeared at all. Insulin was suppressed. Blood was collected for assay (serum S_1). Decompensation of diabetes follows withdrawal of therapy. A second "resistant" blood serum was drawn on 8/9/63 (R_2). Note the evident, but not so striking response, when ditiazanine was re-instarted. A fall of urinary volume and glycosuria was observed after chlorpropamide and phenethylbiguanide therapy. Blood sample (R_3) was drawn before starting therapy and another sample (S_2) obtained 10 days later for immunoassay.

kept on standard diet; the metabolic response although evident was not so striking as observed when the patient was under rice diet. Because of intolerance symptoms exhibited by the patient (nausea) the treatment could not be sustained. Starting Sept. 10 the patient was given chlorpropamide (500mg) and phenethyl ..... biguanide (75mg) daily. A third sample of blood for immunoassay (R_3) was collected immediately before this last period of therapy.

A fall of urinary volume and glycosuria to below 4 liters and 100gm respectively was then observed; a fourth sample of blood was obtained after remission of symptoms (S_2, 9/18/63). Under this treatment the patient was discharged, being revised periodically in Out-Patient Clinic. Recently (August 1966), water diuresis was below 3 liters, glycosuria was absent and no symptoms due to carbohydrate disturbance were present. A retinian hemorrhage occurred a few months ago, the patient being under ophtalmologic care.

Immunoassay methods and results

I - Blood collected during the insulin-resistant phase (R). Assay for serum insulin antibody according to the radio-immunoassay method was performed. Applying amounts greater than 0,2 microgram on paper strip Whatmann N. 1, the insulin-^{131}I plus albumin or mixed with normal serum migrated in the albumin or α_1 - albumin zone after 16 hours of electrophoresis (figs. 10, 11). Mixed with patient's serum the insulin migrated with gamma-globulin .... fraction (fig. 12). Using the McFarlane method for preparation of insulin -^{131}I of high specific activity[16] and applying amounts below 0,2 microgram on strip of Whatmann 3MM filter paper alone or mixed with normal serum the peak remained at origin or moved .... slightly away from the side of application (fig. 13, 14); when mixed with the serum of the patient the peak of activity moved to gamma or beta-gamma globulin interzone (fig. 14) These results were repeatedly confirmed with the same serum and serum from some

other resistant phase.

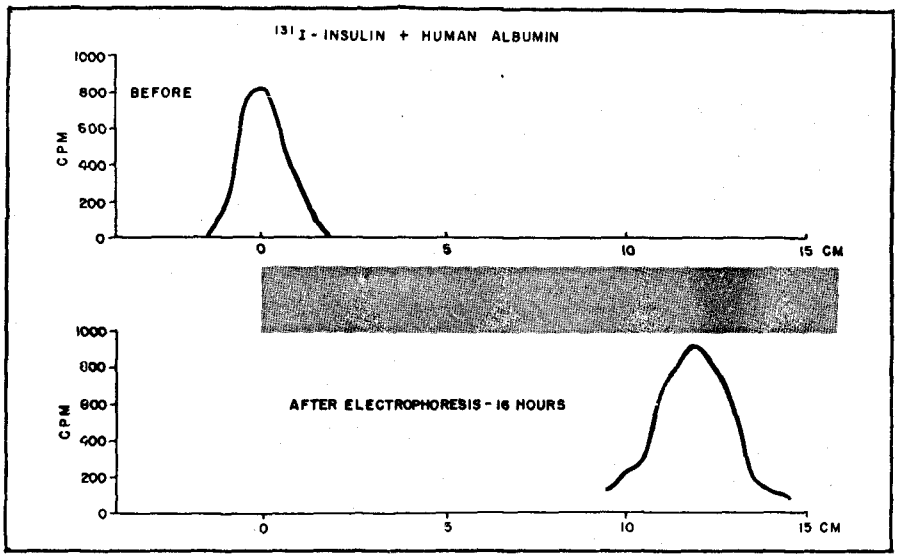


Fig. 10 - Electrophoresis of ^{131}I-Insulin on paper Whatmann nº 1. Gamma activity was measured by an autoscanner before and after 16 hours of electrophoresis. Note that insulin moves toward albumin zone.

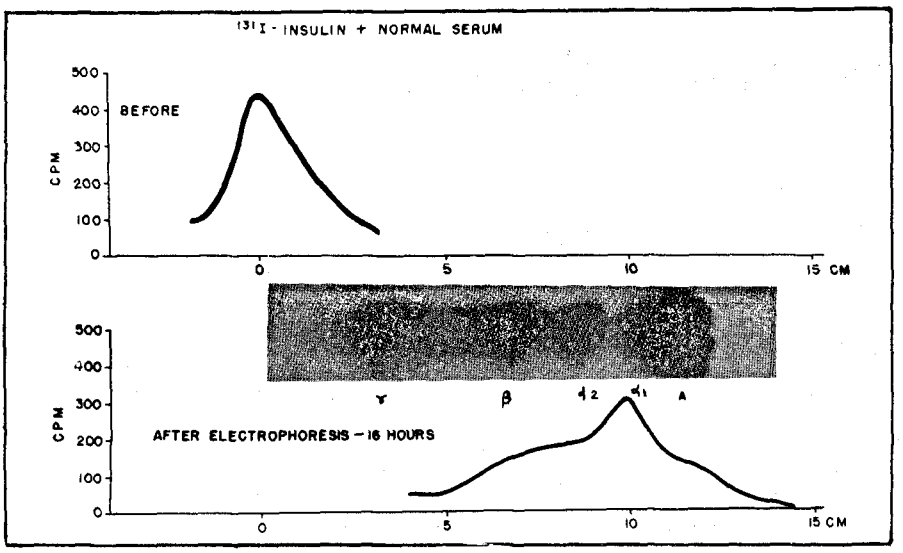


Fig. 11 - Mixed with normal serum the ^{131}I-Insulin migrates in α_1-albumin zone.

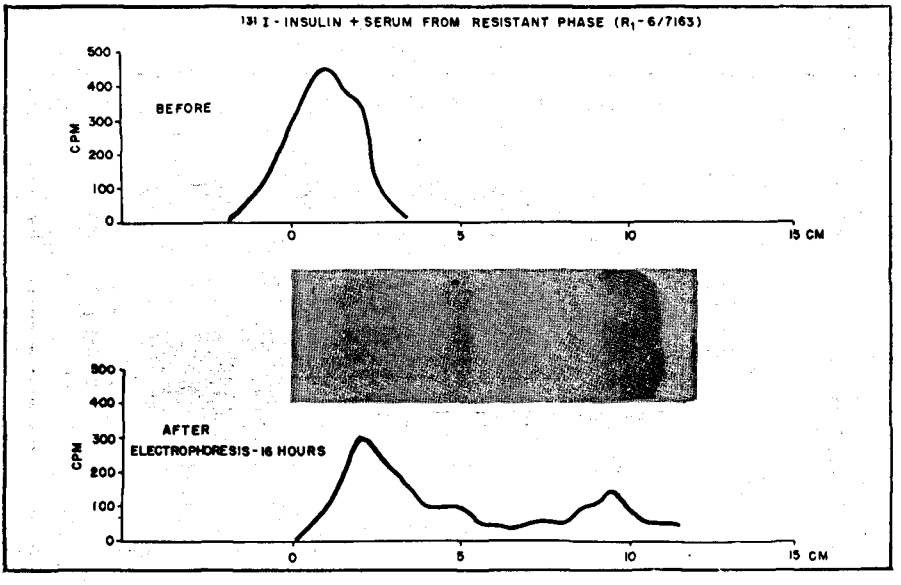


Fig. 12 - Mixed with patients serum the ^{131}I-insulin migrates with gamma-globulin fraction, indicating the presence of anti-insulin antibody. Controls are shown in the figs. 10 and 11.

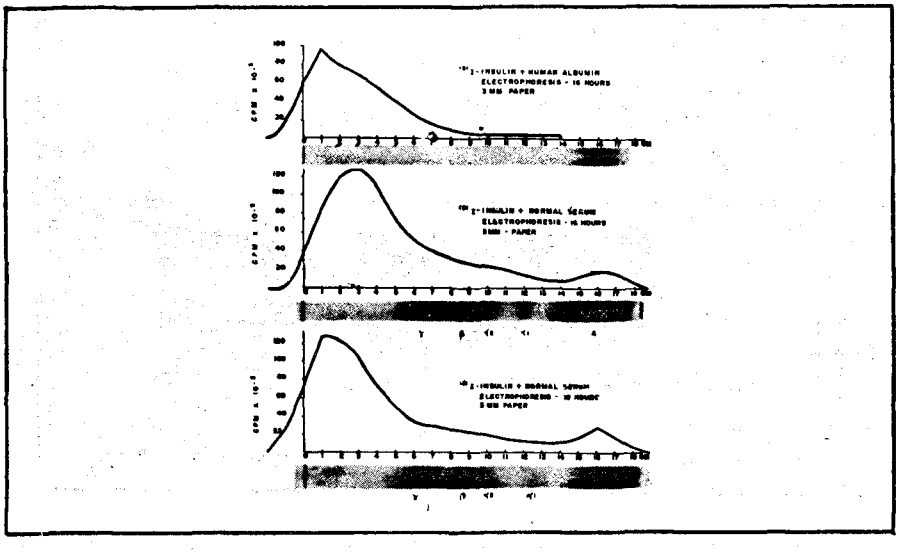


Fig. 13 - Applying ^{131}I-insulin of high specific activity on strip of Whatmann 3MM filter paper alone or mixed with normal serum, the peak of activity remained at origin or moved slightly away from the site of application.

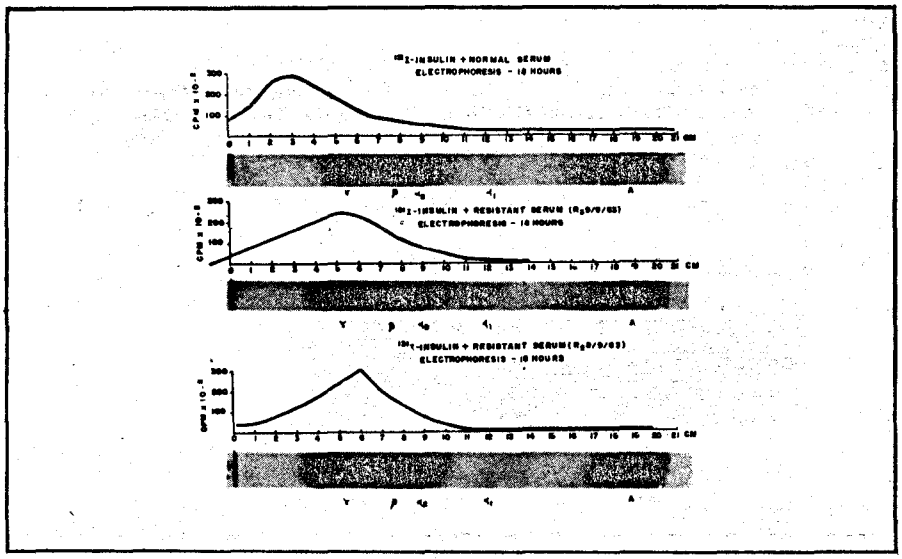


Fig. 14 - Mixed with the serum of the patient, during the apparent resistant phases (R2, R3), the peak of activity moved to gamma or beta-gamma globulin inter zone. Compare with control shown at the top of the fig. 13.

II - Blood collected during insulin-sensitivity phase (S). During the non-resistant phase which occurred after ditiazanine and chlorpropamide-biguanide treatment, both techniques were again employed:

a) Applying amounts greater than 0,2 microgram on strip of Whatmann Nº 1 filter paper for 8 hours electrophoresis, the ^{131}I Insulin mixed with normal serum migrates in the albumin zone (fig. 15); the same was observed with mixture of ^{131}I-Insulin plus non-resistant serum (fig. 16).

b) Applying amounts below 0,2 microgram alone or plus normal serum on strip of Whatmann 3MM filter paper for 16 - 18 hours, the ^{131}I-Insulin remained at the origin or moved away from the site of application, but fixing before gamma-globulin (fig.17). The same was not observed when amounts below 0,2 microgram of ^{131}I

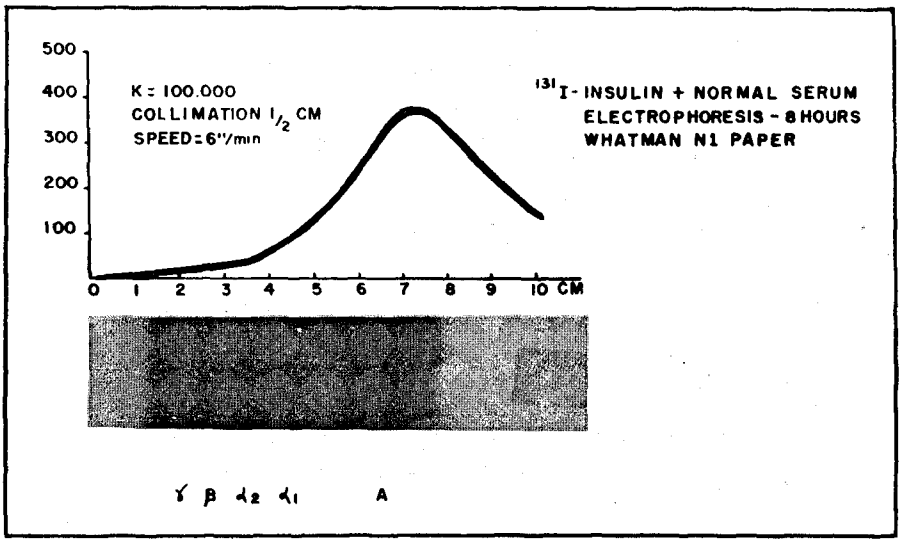


Fig. 15 - Using ^{131}I-Insulin of low-specific activity the peak moves toward albumin zone, when mixing with normal serum.

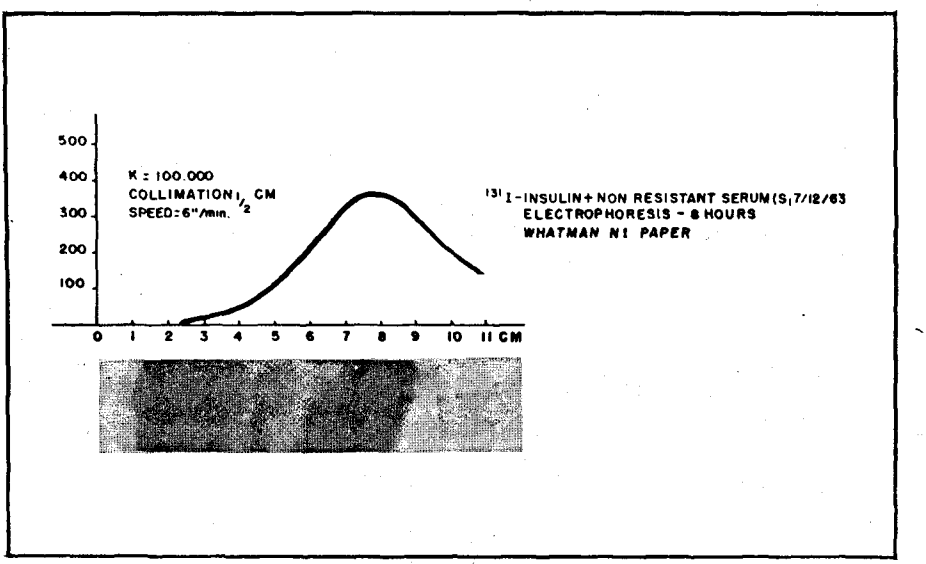


Fig. 16 - Using ^{131}I-Insulin of low-specific activity the peak moves toward albumin zone when mixing with serum from non-resistant phase.

Insulin mixed with serum from a non resistant phase was applied on filter paper. In this case labelled insulin moved in interbeta-alpha-2 or interalpha zone (figs. 17, 18, 19), clearly apart from the gamma or the interbetagamma globulin zone. Figure 20 shows the electrophoretic pattern of ^{131}I-Insulin mixed with serum from a diabetic young woman receiving 60 units of NPH plus 30 units of Regular Insulin: the peak of activity moved in interalpha zone; no discontinuance of therapy was done in this case. Figure 21 shows the electrophoretic pattern of ^{131}I-Insulin mixed with serum from a diabetic young woman receiving 60 units of NPH plus 30 units of Regular Insulin (30-30-25): the peak of activity moved slightly away from the site of application, there being a clear protein free zone between ^{131}I-Insulin radioactivity and the slowest migrating gamma-globulin protein; in this case, the insulin therapy was suppressed 24 hours before the blood collection.

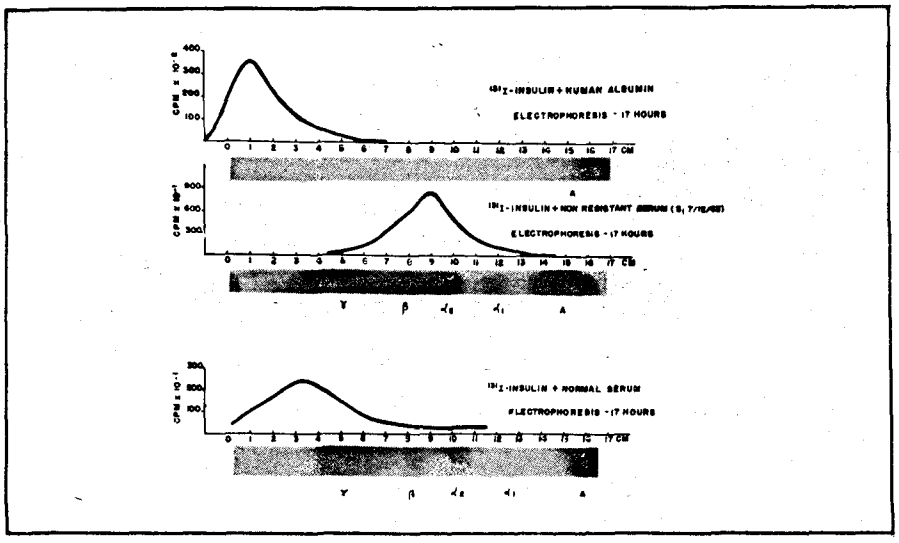


Fig. 17 - ^{131}I-Insulin of high specific activity mixed with serum from non-resistant phase (S_1) moved in interbetaalpha-2 or interalpha zone, clearly apart from the gamma or the interbetagamma globulin zone. The ^{131}I-Insulin alone or plus normal serum remains at the origin or moves away from the site of application, but fixing before gamma-globulin.

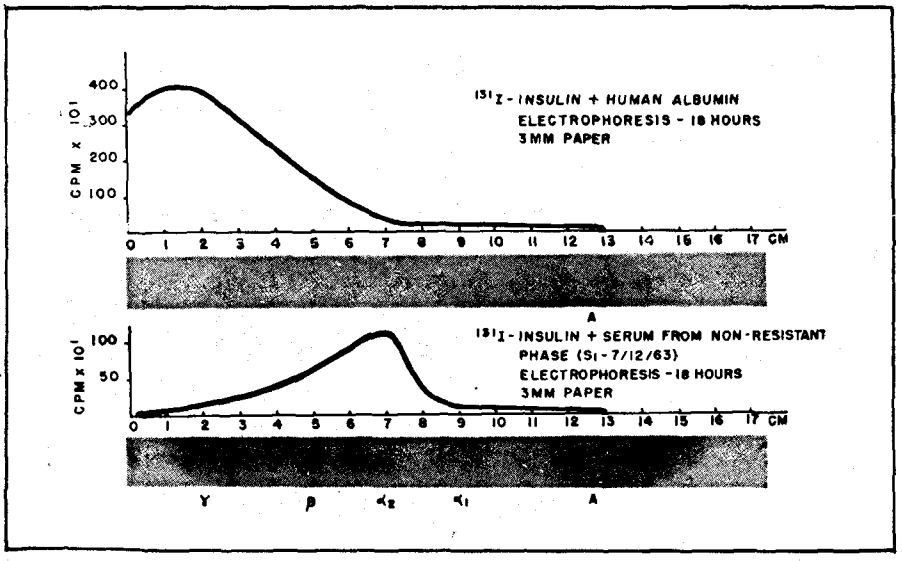


Fig. 18 - Identical result with S_1 serum, in another experiment (see text fig. 17).

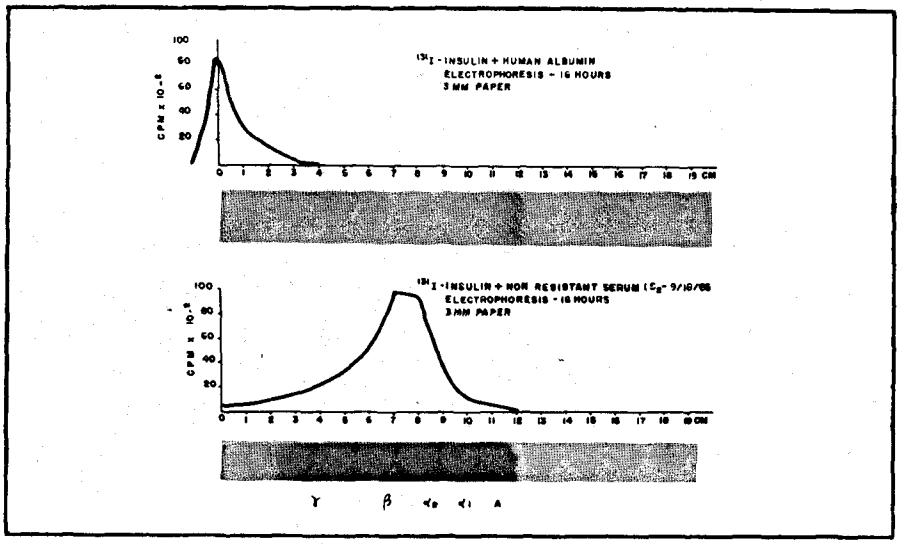


Fig. 19 - The same result with S_2 serum was obtained as compared with S_1 serum, both from non-resistant phase. See text of fig. 17.

DISCUSSION

Apparently the resistance to insulin in the first case (R.P.) could be related to an immunobiological mechanism. Due to the fat that no detectable neutralizing antibody could be evidenced in the circulating blood, the only reasonable suggestion to explain the presence of such mechanism would be the possible neutralization of insulin at cellular level. If this is true the insulin-like activity of the patient serum should be very high during the course of treatment. The first published report indicative of such a possibility, i.e., abnormally high concentration of circulating insulin activity was that of Davidson and Eddleman[17], who produced convulsion in mice by injecting the serum of an insulin-resistant patient. Presland and Todd[18] could later demonstrate a considerable amount of circulating insulin in an unusual case of extreme and prolonged insulin resistance: they showed that insulin resistance was not due to excessive inactivation of insulin in the blood and suggested a "defective response" in the tissues. The electrophoretic pattern showed an elevated proportion of the globulins and, when the insulin requirement diminished, the protein spectrum moved towards normal. The high concentration of blood insulin was clearly due to the presence of the active exogenous insulin and to its persistance in the circulation for a relatively long period. So the insulin would be free and available to exercise its normal effects because it exerted a characteristic hypoglicemic action in the rat and stimulated the formation of glycogen in isolated rat muscle. Presland and Tood suggested that the defect was in the tissues at the site of insulin's action and pointed out that the investigation did nor provide evidence which would help to distinguish the "factors" that might operate at this site to produce insulin resistance. Martin et al.[19], Glass et al.[20] reported case of marked insulin resistance in which no passive transfer or other antibodies to insulin could be demonstrated. Marked resistance to

insulin was reported bu Tyler and Beigelman[3] in a case of diabetic coma in which the patient died in a period of 57 hours in spite of receiving 97.740 units of intravenous insulin. The authors didn't try to use ACTH or corticoids. In this case, Tyler and Beigelman were able to demonstrate the presence of insulin-like activity in the serum of the patient and suggested an end-organ refractoriness.

The resistance to insulin in our first patient could be attributed to the same cause if steroid therapy had not been instituted. However, the striking life-saving effect of cortisol indicates that resistance of this patient could be related to an immunobiological mechanism. It should be noted, however, that two peculiar facts must be taken into account: I) the rapidity of the clinical and metabolic response to the cortisol that occurred in less than 60 minutes and II) the failure to demonstrate the presence of circulating neutralizing antibodies by the available methods. Yet, as will be dicussed later, there are several information pertinent to the clinical and metabolic evolution, which support the conception of the immunological nature of the resistance exhibited by the first patient. To accept this idea we must admit the theoretical possibility, which cannot be excluded or proved, that neutralizing activity takes place at cellular level through a kind of special binding globulin which would be fixed and activated upon surface structure, at the same sites of insulin action. Albeit this insulin-binding protein must be necessarily carried across the circulating blood, it is not active at this level and no complex antigen-antibody would be formed. If this is true, additional amounts of exogenous insulin, no matter how great, will remain free (and theoretically active) but not metabolizable or available to the tissues, due to the blocking of the sites for its action by the complex insulin-insulin-binding proteins fixed at this level. In this way we can explain this extraordinarily high concentration of insulin-like activity of plasma

and prolonged half-life of ^{131}I-Insulin in sone patients with the so called "end-organ refractoriness". Both, exogenous insulin administered and antibody, whose production it stimulates, are kept in the circulation without complexing. This fact explains the impossibility of detecting antibody in serum through direct and indirect methods available. The expected rise in proportion of the globulins as found by Presland and Todd[18], although not specific, is in according with this hypothesis. On the other hand, accept ing this hypothesis e being correct, one can explain the very quick clinical and metabolic response observed in the patient to Cortisol administration. In fact, the steroid could act immediately (minutes), by releasing the antigen antibody complexes from the sites of action of insulin, and more slowly (weeks, months) by reducing and destroying the ability of the tissues for immunoglobulin production. On the other hand, this reasoning being correct, a somewhat dangerous possibility of the appearance of severe episodes of hypoglicemic crisis was to be expected whenever the sites of insulin action have been liberated by corticoid thera py. Probably the sustained infunsion of dextrose solution and marked reduction in insulin administration during the period which followed the first cortisol injection, when anticipating the presence of the greatest excess of exogenous insulin, might have avoided the appearance of hypoglycemia. It should also be considered that insulin is under the continuous action of liver insulinase, as demonstrated by Yalow and Berson[21]. Hypoglycemic bouts occurred at night, on the 14th, 15th and 16th April, when the patient was submitted to continued prednisone therapy and constant doses of insulin. To avoid these crises it was decided to rearrange the time of meals. Up to this time there was to be expected a progressive decrease in the rate of antibody production; the aggravation of metabolic disturbance, although striking after discontinuance of steroid therapy, occurred in fact about 30 hours after the first and second cortisol injection, 2

days after the first and 5 days after the second prednisone therapy withdrawal.

As a matter of fact, one cannot consider the patient as having an endorgan refractoriness, in view of the relatively quick and dramatic response to corticoid therapy. Indeed the efficiency of the glucocorticoid as a lifesaving device could be unequivocally proved in two circunstances: first, when the second intravenous injection of cortisol was followed by an evident improvement in the recurrent ketoacidosis which followed the first dose; second, on those two occasions, when the aggravation of the metabolic disturbances and clinical deterioration of the patient after the discontinuance of prednisone were observed.

Another theoretical possibility of explaining the resistance, still on immunobiological basis, is to hold the binding of insulin to immunoglobulin fixed on the tissues responsible for antibody production. In this case, it is hard to explain the reason for the failure of exogenous insulin to saturate the antibody system despite of increasing amounts of the hormone administered.

The present case is reported just to call attention upon the possibility of occurrence of a probable immunoresistance to insulin without any detectable circulating antibody, thus simulating end-organ refractoriness. In spite of the speculative character of the discussion, it was our intention to try testing a new possibility in the field of immunology. Perhaps only two cases of proved end-organ refractoriness were reported in the literature. The first one is that of Field el al.[6], who reported a young female with uncontrolled diabetes for one year receiving up to 38.000 units of insulin daily in the Hospital, without appreciable effect on her constant hyperglycemia and acetonuria; beef, pork and sheep insulin, phenethyl-biguanide, metahexamide and hydrocortisone were tried without modifying her insulin resistance; her clinical condition remained essentially the same when all

medication was stopped. As long as three months after her last insulin injection, insulin-like activity was detectable in her plasma equivalent to at least 4.000 times that of normal subjects (rat hemidiaphragma technique). In this case it is suggested that diabetes resulted from an apparent failure of peripherical tissues to respond to insulin. The intimate cause for such end organ refractoriness persists unknown. Another case of marked resistance associated with the presence of high insulin-like activity in the plasma was reported by Tucker et al.[8]. Extraordinarily large amounts of insulin were given to the patient (a 25 year old Negro woman), 194.100 units during 48 hours treatment of acidosis and 177,500 during 24 hours at a later date without acidosis. The authors tried the use of prednisone, chlorpropamide and phenformin alone and in combination with insulin, but no effects on glucose utilization were apparent. They suggested that loci of resistance existed beyond the plasma, either at the cell membrane or within the cell. The cause for such a tissue resistance is unknown.

In our second case (I.F.D.F.) neutralizing or blocking antibody could be detected in the blood during the resistant phase (R) with the radioimmunotechnique. The disappearance of the clinical resistance coinciding with the administration of .... ditiazanine iodide [a poor soluble tiazine (S1)] and hypoglycemic drugs (S2) was followed by a striking change in the electrophoretic pattern of labelled insulin. In fact, I) when a mixture of ^{131}I-Insulin highly concentrated and of low specific activity with normal serum and serum from a non-resistant phase (S1, S2) was applied on filter paper, the radioactivity moved with albumin. This new pattern suggested that the ditiazanine iodide (serum S1) or hypoglycemic drugs (serum S2) were able to destroy or inactivate the antibody or simply to ptomete the cleavage of the insulin-antibody complex; II) when tracer amounts of ^{131}I - Insulin mixed with serum from the non-resistant phase (S) run on Whatmann Nº 3MM paper the peak occurred repeatedly in interbeta-

alpha or interalpha zone (fig. 17, 18, 19), whereas mixed with normal serum it remained at or near the origin, there being a protein-free zone between the ^{131}I-Insulin radioactivity and the slowest migrating gamma-globulin protein (fig. 17, 18, 19). The occurrence of the peak between the beta and alfa-2 proteins was eventually observed by Berson et al.[12], when the paper was soaked with insulin at origin prior to electrophoresis, and it is due to the saturation of the binding sites of the paper. Theses results indicate that in presence of high concentration of free insulin in plasma of the patient during treatment, the labelled insulin moves far from the origin. Thus, besides confirming the absence of active antibody as demonstrated by the former method (I) the latter one (II) led to assume that, during treatment, the plasma of the patient must contain a very high concentration of insulin which is liberated from the complex antigen-antibody .... through the action of the drugs. In order to confirm this assumption, serum from two insulin sensitive diabetics receiving relatively high doses of insulin were collected. From the first one, a young female diabetic receiving 60 units NPH insulin plus 30 units of Regular Insulin, blood was drawn without discontinuance of therapy. The electrophoretic pattern was entirely superposable to those obtained in experiment II when tracer amounts of ^{131}I-Insulin were used (fig. 20). In the second case,when insulin therapy was discontinued and blood collected 24 hours after the last insulin injection, the electrophoretic pattern was similar to that obtained with normal serum (fig. 21). Presumably in the latter case the concentration of insulin in the plasma was not sufficiently high to permit saturation of the paper.

In favour of the hypothesis that exogenous insulin could be liberated from the antigen-antibody complexes through the action of the drugs is the experiment of Hasselblatt[22], who was well succeeded in promoting the liberation of insulin with ..... tolbutamide in vitro. Indeed, this binding could be prevented by

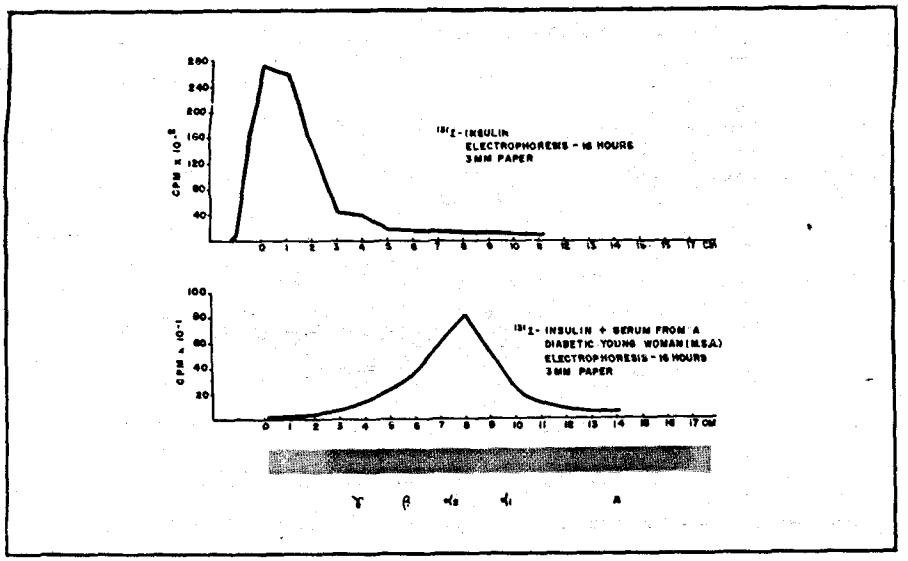


Fig. 20 - Electrophoretic pattern of ^{131}I-Insulin mixed with serum from a diabetic young woman receiving 60 units NPH plus 30 units of Regular Insulin. No discontinuance of therapy was done in this case. Note that the peak of activity moved in inter-alpha zone.

addition of tolbutamide. On the supposition that hypoglycemic drugs could promete a similar action in human resistance to insulin, the patient was given chlorpropamide to inhibit the binding of exogenous insulin and decrease the resistance of the patient to treatment. Biguanide was given in conjunction with chlorpropamide to increase glucose uptake by peripheral tisues and decrease hepatic gluconeogenesis. This treatment was well succeeded, yielding satisfactory and sustained compensation for diabetes, independently of the administration of exogenous insulin. The striking effect of chlorpropamide on the glycosuria in absence of insulin administration must be due to its pharmacological action on beta cell, increasing the endogenous production of insulin, which presumably does not cross-react with gamma globulin anti-exogenous insulin. The study of the effect in vitro of ditiazanine iodide, and chlorpropamide upon antigen-

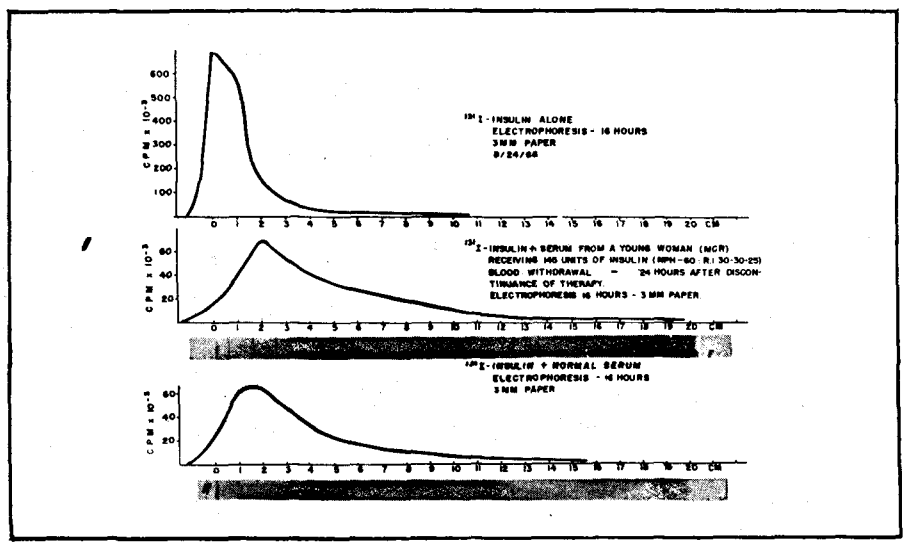


Fig. 21 - ^{131}I-Insulin mixed with serum from a diabetic young woman receiving 60 units NPH plus 30 units of Regular Insulin. In this case the insulin therapy was suppressed 24 hours before the blood collection. Note that the peak of activity moved slightly away from the site of application, there being a clear protein-free zone .... between ^{131}I-insulin radioactivity and the slowes migrating gamma-globulin protein.

antibody complex presumably present in the patient's serum, was not well succeeded owing to the fact that incubation of these drugs with ^{131}I-Insulin alone or plus normal or resistant serum has always been followed by molecular lesion, promoting the appearance of a multitude of peaks of activity throughout the electrophoretic pattern. The marked and persistent reduction of the urinary daily volume which was out of proportion with diabetic state, could be explained by the antidiuretic efect of hypoglicemic drugs on true diabetes insipidus[23].

In an attempt to confirm this hypothesis the patient was admitted to the Hospital on March 17, 1967. She was put on standard diet for diabetes and 50 units NPH insulin daily. The oral ... hypoglycemic drugs she was receiving before admission were kept unchanged (250mg chlorpropamide, 75mg biguanide). Withdrawal of oral hypoglycemic drugs on March 28 was followed by a marked

rise in urinary volume, in spite of maintainance of insulin administration. Increase in water diuresis was out of proportion with the glycosuria change. As can be seen in figure 22, administration of chlorpropamide alone was followed initially by a dramatic fall in urinary volume without corresponding change in glycosuria. Insulin administration (April 25) followed by chlorpropamide withdrawal (May 2) did'nt prevent the rising in urinary volume. Water diuresis decreased again after chlorpropamide resumption without corresponding change in total glycosuria which persisted inaltered for about two weeks.

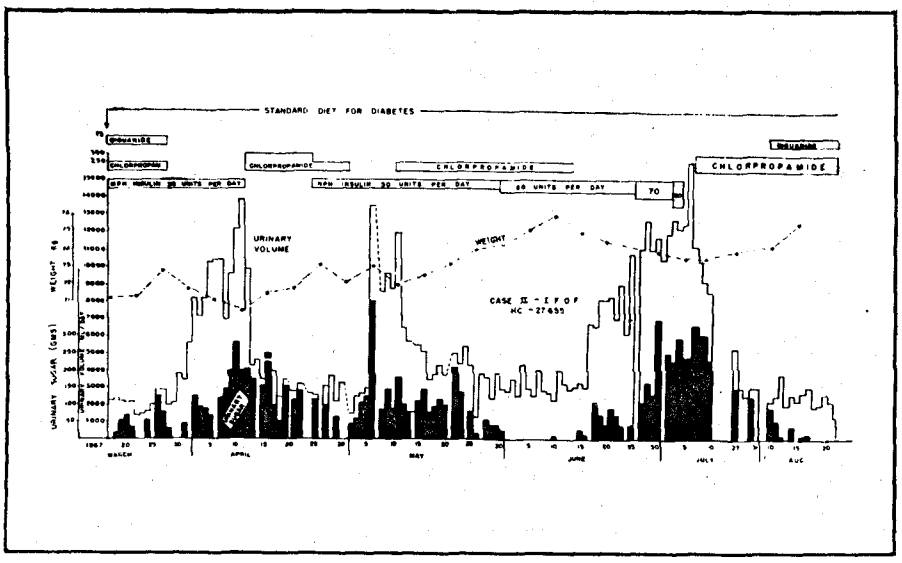


Fig. 22 - See text.

The antidiuretic effect of chlorpropamide was confirmed consecutively in April, May and June. Resistance against insulin treatment was clinically observed during May and June, when increasing amounts of insulin up to 80 units daily were clearly insufficient to reduce the glycosuria, which increased markedly after chlorpropamide with drawal. The striking effect of chlorpropamide alone and in conjuction with biguanide could be observed

in July and August when the glycosuria disappeared at all and urinary volume came down below 3 liters per day, just before discharge.

RESUMO

Estudos imunobiológicos em dois casos de diabetes insulino-resistentes. Terapêutica corticóide bem sucedida em um caso com acidose progressiva, sem circulação detectável de anticorpos. Efeito antidiurético da clorpropamida em outro caso com diabete insípido associado.

São relatados os estudos clínicos e imunológicos de dois casos de marcada resistência à insulina. No primeiro caso não foi possível demonstrar, pelas técnicas disponíveis, a presença de anticorpos circulantes. Entretanto, o nítido efeito benéfico da hidrocortisona sôbre a evolução da acidose diabética tornou possível relacionar a resistência à insulina exibida pelo paciente, a um mecanismo imunobiológico.

No segundo caso foi possível evidenciar a existência de anticorpo bloqueador ou neutralizador (contra a insulina exógena), durante a fase de resistência, pela técnica rádio-imunobiológica. O desaparecimento clínico da resistência coincidente com a administração do iodeto de ditiazanina (diazina de pouca solubilidade) e de drogas hipoglicemiantes, foi seguido por marcada alteração do padrão eletroforético da insulina radioativa. Êste nôvo padrão de comportamento eletroforético forneceu indícios de que ambas as drogas eram capazes de destruir ou inativar o anticorpo ou, simplesmente, promover a clivagem do complexo insulina-anticorpo, assim diminuindo ou fazendo desaparecer a resistência à ação da insulina exógena. Neste segundo caso foi comprovada também a existência de associação de diabetes insipidus com o diabetes resistente à insulina. A marcada redução da diurese, fora de proporção com a glicosúria, foi explicada através do efeito antidiurético da clorpropamida sôbre o diabetes insipidus.

RÉSUMÉ

Études immunobiologiques en deux cas de diabète insulino-résistants. Succès de la thérapie corticoïde dans un cas avec acidose progressive, sans circulation détectable d'anticorps. Effet antidiurétique de la chlorpropamide dans un autre cas avec diabetes insipidus associé.

Les auteurs communiquent des études cliniques et immunologiques sur deux cas de résistance marquée à l'insuline. Dans le premier cas il n'a pas été possible, avec les techniques disponibles, de détecter la présence d'anticorps circulants. Pourtant, l'effet nettement favorable de la hydrocortisone sur l'evolution de l'acidose diabétique permet de relationner l'insulino-résistance présentée par le patient à un mécanisme immunobiologique.

Dans le deuxième cas, on a pu évidencer, par la technique radioimmunobiologique, l'existance d'un anticorps bloquant ou neutralisant l'insuline exogène, pendant la phase de résistance. La cessation clinique de la résistance, coincidant avec l'administration de iodure de ditiazanine (diazine peu soluble) et de drogues hypoglycémiantes, fut suivie d'une remarquable altération du standard électrophorétique de l'insuline radioactive. Ce nouveau standard de comportement électrophorétique a fourni des indices dans le sens que les deux drogues seraient capables de détruire ou d'inactiver l'anticorps, ou, simplement, de produire le clivage du complexe insuline-anticorps, ce qui faisait diminuer ou disparaitre la résistance à l'action de l'insuline exogène. Dans ce deuxième cas, on constata aussi l'existance d'une association de diabetes insipidus avec le diabète insulino-résistant. La remarquable réduction de la diurèse, hors de proportion avec la glycosurie, est expliquée par l'effet antidiurétique de la chlopropamide sur le diabetes insipidus.

ACKNOWLEDGMENT

This work was performed in the Radiobiologic Divison of the Institute of Atomic Energy, S.Paulo, SP, Brazil, in cooperation with the Metabolic and Endocrinologic Unit of the Clinics Hospital

of the Faculty of Medicine, U.S.P. The authors wish to thank Mr. Emilio Engelstein (biochemist), Mrs. Atsuko Nakasone (pharmacist) and Mr. Günther Höxter (chemist) for their valuable technical assistance.

REFERENCES

1. Mauriac, P. & Aubertin, E.: Insulino-résistance et diabetes par neutralization, Presse Medicale 34: 1633, 1926.

2. Root, H.F.: Insulin resistance and bronze diabetes, N.England J.Med. 201: 201, 1929.

3. Tyler, D.R. & Beigelman, P. M.: Insulin-resistant coma , Diabetes 9: 17, 1960.

4. Wayburn, E. & Bechh, W:Insulin resistance in dietes mellitus, J. Clin. Endocrinol. 2: 511, 1942.

5. Wiener, H.J.: Diabetic coma requiring an unprecedented amount of insulin, Am. J.M. Sc. 196: 211, 1938.

6. Field, J.B. Weinberg, A., Johnson, P. & Spoont, S.: An unusual case of diabetes mellitus with increased pancreatic production of insulin, J. Clin. Invest, 38: 1003, 1959.

7. Colleus, W.S. & Banowitch, M.M.: Insulin-resistance:report of an unusual case with requirements up to 7.840 units in 24 hours, Metabolism 4: 355, 1955.

8. Tucker, W.R., Klinik D., Goltz, F., Zalme E. & Knowles,H.C. J.: Diabetes 13: 395, 1964.

9. Banting, F.G., Franks, W.R. & Gairns, S.: Anti-insulin activity of serum of insulin - trated patient, Am. J. Phychiat 95: 562, 1938.

10. De Filippis, V. & Iannaccone, A.: Insulin-neutralizing activity of gamma-globulins derived from the serum of an insulin-resistant patient, Lancet 1: 1192, 1952.

11. Berson, S.A. & Yallow, R.S.: Quantitative aspects of the reaction between insulin and insulin-binding antibody. Berson, S.A. and Yallow, R.S., J. Clin. Invest. 38: 1996, 1959.

12. Berson, S.A., Yallow, R.S. Bauman, A., Rothschild M.A. & Newerly, K.: Insulin-^{131}I metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin - treated subjects, J. Clin. Invest. 35: 170, 1956.

13. Burrows, S.A., Peters, T. & Lowell, F.C.: Physical binding of insulin by gamma-globulins of insulin-resistant subjects, J. Clin. Invest. 36: 393, 1957.

14. Banks, H.H., Seligman, A.M. & Fine, J.: The effect of hyaluronidase on the absorption of parenterally administered radioactive proteins in the dog. J. Clin. Invest. 28: 548, 1949.

15. Kallee, E.: Über ^{131}J-signiertes Insulin II, Klin. Wochschr. 32: 21, 1954.

16. Mc Farlane, A.S.: Efficient trace labelling of proteins with iodine, Nature (London) 182: 53, 1958.

17. Davidson, J.K. & Eddleman, E.E.: Insulin Resistance. Review of the literature and report of a case associated with carcinoma of the pancreas, Arch. int. Med. 86: 727, 1950.

18. Presland, J.R. & The Late C.M. Todd: An investigation of prolonged insulin resistance in a case of diabetes mellitus , Quart. J. Med. 25: 275, 1956.

19. Martin, W.P., Martin, H.E., Lyster, R.W. & Strouse, S: Insulin

resistance, critical survey of literature with report of case, J. Clin. Endocrinol. 1: 387, 1941.

20. Glass, W.I., Springarm, C.L. & Pollack, H.: Unusually high insulin requirements in diabetes mellitus, Arch. int.Med. 70: 221, 1942.

21. Yallow, R.S. & Berson, S.A.: Apparent inhibition of liver ... insulinase activity by serum and serum fractions containing insulin-binding antibody, J. Clin. Invest. 36: 648, 1957.

22. Hasselblatt, A.: Liberation of insulin bound to serum protein by tolbutamide, Metabolism XII: 302, 1963.

23. Arduino, F., Ferraz, F.P.J. & Rodrigues, J.: Antidiuretic action of chlorpropamide in idiopathic diabetes insipidus,The J. of. Clin. Endocrinol, and Metab. 26: 1325, 1966.